University of Córdoba

Analytical Chemistry Department

# INNOVACIONES EN TÉCNICAS DE MICROEXTRACCIÓN CON AGITACIÓN INTEGRADA

## *INNOVATIONS IN MICROEXTRACTION TECHNIQUES WITH INTEGRATED STIRRING*

**DOCTORAL THESIS**

**Mercedes Roldán Pijuán**

Córdoba, 2015

TITULO: *Innovaciones en técnicas de microextracción con agitación integrada*

AUTOR: *Mercedes Roldán Pijuán*

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# INNOVACIONES EN TÉCNICAS DE MICROEXTRACCIÓN CON AGITACIÓN INTEGRADA

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*Trabajo presentado para aspirar al Grado de Doctor en Ciencias*

*LA DOCTORANDA*


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**CERTIFICAN:**

Que la Tesis Doctoral "**INNOVACIONES EN TÉCNICAS DE MICROEXTRACCIÓN CON AGITACIÓN INTEGRADA**" ha sido desarrollada en los laboratorios del Departamento de Química Analítica de la Universidad de Córdoba (España), y en el Departamento de Farmacia de la Universidad de Oslo (Noruega), y que, a nuestro juicio, reúne todos los requisitos exigidos a este tipo de trabajo.

Que Mercedes Roldán Pijuán es la primera autora de todos los trabajos científicos presentados en esta Tesis Doctoral. De acuerdo con la normativa de esta Universidad y los acuerdos internos del Grupo de Investigación, el primer autor es el responsable de la realización del trabajo experimental y de la producción del manuscrito. Además, ha participado activamente en las reuniones periódicas con los supervisores para evaluar y discutir los resultados obtenidos durante la Tesis Doctoral.

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en la ciudad de Córdoba, a 9 de Febrero de 2015.

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**Rafael Lucena Rodríguez**

**Mº Soledad Cárdenas Aranzana,** Full Professor of Analytical Chemistry Department of the University of Córdoba; **Rafael Lucena Rodríguez**, Professor of Analytical Chemistry Department of the University of Córdoba, in quality of supervisors of the Doctoral Thesis of Mercedes Roldán Pijuán,

**CERTIFIES THAT:**

The Doctoral Thesis entitled **"INNOVATIONS IN MICROEXTRACTION TECHNIQUES WITH INTEGRATED STIRRING"** has been developed in the laboratories of the Department of Analytical Chemistry of the University of Córdoba (Spain) and in the School of Pharmacy at Oslo University (Norway), and, according to our judgement the thesis fulfills all the requirements of this type of scientific work.

Mercedes Roldán Pijuán is the first author of all the scientific articles developed during the experimental phase of the Thesis. According both to the University rules and internal agreements in our research group, the first author of a paper is the full responsible for the implementation of the experimental work and also to produce the first draft of the paper. In addition, she has also actively participated in the meetings with the supervisors to check and discuss the progress of the doctoral work.

Córdoba, 9 February 2015.

**Mº Soledad Cárdenas Aranzana**

**Rafael Lucena Rodríguez**

**TÍTULO DE LA TESIS: Innovaciones en técnicas de microextracción con agitación integrada**

**DOCTORANDO/A: Mercedes Roldán Pijuán**

## INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La licenciada Mercedes Roldán Pijuán cursó con distinción la Licenciatura de Ciencias Ambientales y brillantemente los estudios del máster en Química Fina Avanzada. En el año 2010 accedió a una beca de formación de profesorado universitario para la realización de la Tesis Doctoral, cuya Memoria se presentará para su defensa el próximo mes de febrero bajo la modalidad de doctorado internacional y como compendio de publicaciones.

La temática de la misma ha sido el desarrollo de herramientas de microextracción basadas en la integración de la agitación y la extracción en el mismo dispositivo. Las metodologías propuestas comprenden tanto la microextracción en fase sólida como líquida, empleando medios de extracción convencionales, nanoestructurados y nuevas fases extractivas.

El trabajo experimental realizado se ha materializado en seis artículos científicos, publicados o enviados para su publicación a revistas especializadas del área. Se han publicado además dos capítulos de libro y un artículo de divulgación. La doctoranda ha asistido a siete congresos nacionales e internacionales presentando un total de 12 comunicaciones en formato oral, flash o cartel. A lo largo de estos años, ha adquirido formación en técnicas de microextracción, cromatografía de líquidos y de gases, tratamiento de muestras de agua y fluidos biológicos para la determinación de distintas familias de compuestos. Además de las competencias técnicas inherentes al trabajo en el laboratorio, ha demostrado iniciativa, capacidad para la resolución de problemas, planificación y dirección del trabajo a investigadores noveles así como para la innovación en la investigación. De hecho, ha llevado a cabo la supervisión de un trabajo de investigación de una investigadora visitante de la Universidad de Granada, que se materializó en un artículo científico publicado en la revista Journal of Chromatography A.

Con objeto de poder optar a la mención de doctorado internacional, ha realizado una estancia de tres meses en el Departamento de Farmacia de la Universidad de Oslo (Noruega) bajo la dirección de los profesores S. Pedersen-Bjergaard y A. Gjelstad. El trabajo de investigación desarrollado ha sido aceptado para su publicación en la revista Analytical and Bioanalytical Chemistry.

Finalmente, ha colaborado en la impartición de clases prácticas en los Grados de Química y Ciencias Ambientales durante los cursos académicos 2013-14 y 2014-15. De esta forma ha adquirido las competencias docentes asociadas a la finalidad de la beca disfrutada.

Por todo ello, la tesis doctoral reúne a nuestro juicio los requisitos de calidad y novedad exigibles a este tipo de trabajo, por lo que se autoriza la presentación de la misma.

Córdoba, 9 de Febrero de 2015

Firma del/de los director/es

**Fdo.: Mº Soledad Cárdenas Aranzana**

**Fdo.: Rafael Lucena Rodríguez**

## MENCIÓN DOCTORADO INTERNACIONAL

Mediante la defensa de esta Memoria de Tesis Doctoral se pretende optar a la obtención de la Mención de "Doctorado Internacional" habida cuenta de que la doctoranda reúne los requisitos para tal mención (R.D. 99/2011, de 28 de Enero):

1) Cuenta con los informes favorables de dos doctores pertenecientes a instituciones de Enseñanza Superior de países distintos a España.

2) Uno de los miembros del tribunal que ha de evaluar la Tesis pertenece a un centro de Enseñanza Superior de otro país distinto a España.

3) Parte de la defensa de la Tesis Doctoral se realizará en una lengua distinta de las lenguas oficiales en España.

4) La doctoranda ha realizado una estancia de tres meses en el Departamento de Farmacia de la Universidad de Oslo (Noruega) gracias a la concesión de una ayuda para estancias en el extranjero asociada a una beca de Formación del Profesorado Universitario del Ministerio de Educación, Cultura y Deporte, que ha contribuido a su formación y permitido desarrollar parte del trabajo experimental de esta Memoria.

Agradezco al Ministerio de Educación, Cultura y Deporte la concesión de una beca de Formación del Profesorado Universitario (FPU) que ha hecho posible mi dedicación a este trabajo durante los últimos cuatro años.

I would like to thank Prof. S. Pedersen-Bjergaard and Prof. A. Gjelstad for giving me the opportunity to work at the School of Pharmacy of Oslo (Norway). I am deeply grateful for their continued support and the time devoted to me.

Thanks to Léon and Trine for their kindness. Thanks to all my laboratory mates for their valuable help: Cecilie, Cecilia, Siri H., Siri V., Chuixiu, Marthe, Knut, Lars Erik and Silje. I have really good memories of my time living in Norway and this is mainly because of my favourite Norwegian lab mates Cecilie and my Italian girl, Cecilia. I will never forget you.

Tusen takk!

I would like to thank Prof. A. Kabir and Prof. K.G. Furton from the University of Florida (EEUU) for the common project in which both research group were involved. Thank you for sharing with us your treasure, the so-called fabric phase sorptive extraction media.

Me gustaría también tener unas palabras para el Departamento de Química Analítica de la Universidad de Granada, con los que he tenido la gran suerte de hacer una colaboración durante mi Tesis Doctoral. A los profesores Alberto Zafra, Oscar Ballesteros y Alberto Navalón por su entrega al trabajo. A la ya Doctora Rocío que vino de estancia a nuestro grupo de investigación, por el gran esfuerzo que realizó.

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# OBJETO

*AIM*

La etapa de tratamiento de muestra es, de entre las tres que conforman los procesos de medida (bio)químicos, la que más influencia tiene en la calidad de la información obtenida. Esto se debe fundamentalmente a que hay que adaptarla a la muestra que se analiza, a los analitos que se determinan y a la técnica instrumental seleccionada para tal efecto. En general, las técnicas de tratamiento de muestra juegan un papel importante en la mejora de la sensibilidad y selectividad de las determinaciones a través de la preconcentración de los analitos y de su aislamiento de la matriz de la muestra.

En este contexto, uno de los retos que en la actualidad presenta la Química Analítica, es el avance e innovación en las técnicas de tratamiento de muestra, y su evolución hacia metodologías más sencillas, automatizadas y miniaturizadas. Esta evolución ha permitido la aparición de las técnicas de microextracción, tanto en fase sólida como en fase líquida, que se han consolidado como herramientas útiles para el analista a la hora de resolver problemas analíticos de diferente índole.

Los factores termodinámicos y cinéticos son cruciales a la hora de definir la eficiencia de una técnica de microextracción. Desde el punto de vista termodinámico, una extracción es un proceso de equilibrio químico entre fases, estando dicho equilibrio regido por una constante de distribución. Por otro lado, la cinética de la extracción influye en el tiempo que tarda en alcanzarse dicho equilibrio y viene determinada por la difusión de los analitos desde el seno de la muestra hacia una fase extractiva. De esta manera, la superficie de contacto entre ambas fases es clave. Otro factor que desempeña un papel fundamental en la mejora de la cinética de la extracción es la agitación. Generalmente se requieren largos tiempos para alcanzar el equilibrio, así que una forma de favorecer esta difusión es introduciendo agitación en el sistema. Esta agitación puede llevarse a cabo mediante la utilización de un elemento externo o mediante la integración de éste en la unidad de extracción.

Teniendo en cuenta lo anteriormente expuesto, el objetivo genérico de la Tesis Doctoral que se presenta en esta Memoria se centra en el desarrollo de metodologías analíticas en el

ámbito de la microextracción en fase sólida (Bloque III) y en fase líquida (Bloque IV). Para ello, se proponen y caracterizan nuevas unidades de extracción con agitación integrada que faciliten la separación, preconcentración y detección de los analitos diana. Se trata, en definitiva, de desarrollar nuevas herramientas que presenten un valor añadido a las ya existentes y que mejoren las propiedades analíticas de los procesos de medida (bio)químicos. Dicho objetivo genérico, se ha materializado en los siguientes objetivos concretos:

- Desarrollo de metodologías innovadoras basadas en formatos que integren la extracción y la agitación en el mismo dispositivo.
- Diseño de unidades de microextracción aptas para llevar a cabo el análisis de fármacos en biofluidos que presenten disponibilidad limitada.
- Evaluación del potencial y la versatilidad de distintos materiales como soporte para llevar a cabo procesos de microextracción.
- Evaluación del potencial de nuevos materiales sorbentes, como los nanocuernos de carbono y las fases extractivas sintéticas.
- Desarrollo de metodologías analíticas basadas en el uso de estos materiales sorbentes para la preconcentración y aislamiento de compuestos orgánicos y fármacos en muestras ambientales y biológicas.
- Aplicación de las herramientas propuestas a la resolución de problemáticas en los ámbitos medioambientales y/o clínico-farmacéutico.

*Sample treatment is, among the three steps that are involved in a (bio) chemical process, the one that has the greatest influence on the quality of the obtained information. This is mainly because the sample preparation should be tailored to the sample, the analytes and the selected instrumental technique for the particular purpose. In general, sample treatment techniques play an important role in improving the sensitivity and selectivity of the determinations by means of the analyte preconcentration and isolation from the sample matrix.*

*In this context, one of the main challenges of Analytical Chemistry is the innovation in separation techniques, with a clear trend to simplification, automation and miniaturization. This evolution has lead to the appearance of novel solid and liquid microextraction techniques, which have been consolidated as useful tools for solving analytical problems of different nature.*

*The thermodynamic and kinetic factors are crucial in defining the efficiency of a microextraction technique. From the thermodynamic point of view, an extraction process is defined as chemical phase equilibrium, governed by a distribution constant. On the other hand, the kinetic of the extraction affects the time it takes to achieve this equilibrium and it is determined by the diffusion of the analytes from the bulk solution to an extractive phase. Thus, the contact surface area between both phases is of a great importance. Another factor that plays a pivotal role in improving the kinetic of the extraction is the stirring. Generally, extraction techniques occur under diffusion-controlled conditions and longer extraction times are required to reach the equilibrium. For this reason, one way to increase diffusion rate is through the stirring. This stirring may be performed by use of an external device or by integrating extraction and stirring in the same unit.*

*Considering the above mentioned, the general aim of this Doctoral Thesis is the development of analytical tools in the solid phase (Section III) and liquid phase microextraction field (Section IV). For this purpose, novel extraction devices with integrated stirring are proposed in such a way that they help to simplify the isolation,*

*preconcentration and detection of the target analytes. Briefly, the main aim is focused the development of new and improved microextraction concepts and techniques that enhance the analytical properties of (bio)chemical measurement processes. This general aim was addressed in the following specific objectives:*

- *To develop new analytical methodologies based on integrated extraction/stirring devices.*
- *To design microextraction devices for rapid and selective isolation of drug substances from limited-volume biological fluids.*
- *To assess both the potential and versatility of different materials as support to carry out microextraction processes.*
- *To evaluate the potential of new sorbent materials such as carbon nanohorns and fabric phase sorptive extraction media.*
- *To develop analytical methodologies based on the use of these sorbent materials for the preconcentration and isolation of organic compounds and drugs in environmental and biological samples.*
- *To apply the proposed analytical tools to solve problems in the environmental and/ or clinical-pharmacological field.*

# INTRODUCCIÓN

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# Microextraction techniques based on the combination of agitation and extraction in the same device

*Accepted, 2015*

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The efficiency of a given microextraction technique relies on both thermodynamic and kinetics factors. The first one defines the maximum amount of analyte that can be extracted while the latter describes the rate at which the extraction equilibrium is reached. The stirring of the sample during extraction is a well-known kinetic variable that positively affects the diffusion of the analytes between the donor and acceptor phases. Although stirring/agitation is a critical and optimizing variable in most of the microextraction techniques, only a few of them integrate stirring within the extraction device. The so-called stirring/extraction integrated microextraction techniques present a high potential and they have been the focus of a deep research in the past decade. This encyclopedia article is focused on the description of the main research milestones in this development, giving a wide overview of the potential and applicability of these microextraction techniques.

***Keywords***: *Extraction/agitation integrated techniques; Microextraction; Sample preparation.*

# I.1. MICROEXTRACTION TECHNIQUES BASED ON THE COMBINATION OF AGITATION AND EXTRACTION IN THE SAME DEVICE

## 1. INTRODUCTION

Extraction techniques are widely employed in the analytical chemistry context to improve basic analytical properties such as selectivity and/or sensitivity. Those procedures are intended to isolate the analytes from a complex sample and to preconcentrate them in a lower extraction volume [1]. The past years have witnessed an exponential research and the development in this context is based on three different trends, namely: the development of new procedures, the use of novel materials as extractants or coadjutants, and the application of the developed techniques to solve hundred of analytical problems in the biomedical, environmental and food quality context.

The yield of an extraction procedure depends on two main factors, kinetic and thermodynamic that should be jointly considered in any optimization process [2]. The distribution constant, which defines the partitioning equilibrium of a given analyte between the donor and acceptor phase, is the main thermodynamic variable which can be tuned by selecting the appropriate solid or liquid acceptor phase. All the parameters that affect this constant (such as the ionic strength, the use of secondary reactions, the pressure or the temperature) can be considered as thermodynamic variables although some of them (ionic strength and temperature, especially) may have kinetics connotations. However, a high distribution constant does no guarantee a good extraction performance if the extraction rate is too low. Therefore, kinetic aspects should be considered bearing in mind that the main aim of a technique is to achieve a good extraction yield in a reasonable time.

The influence of several factors on the extraction kinetics has been deeply investigated in the past decades. In this sense, the dependence of the extraction rate with the

donor/extractant contact area is well known and it has served to develop new techniques such as dispersive solid-phase extraction or dispersive liquid-liquid microextraction (DLLME). In addition, the use of stirring to facilitate the transference of the analyte from the donor phase to the acceptor one is a constant in every extraction procedure.

Stirring of the sample can be developed using an external element or integrating this element in the extraction device. The latter approach, which has demonstrated superior performance compared to the first one, has been the subject of investigation for a number of research groups all over the world [3]. This encyclopedia article is focused on the description of the main devices developed to date, describing them and presenting their potential.

## 2. STIR BAR SORPTIVE EXTRACTION

The reference microextraction technique based on the combination of agitation and extraction in the same device is, no doubt, the stir bar sorptive extraction (SBSE), proposed in 1999 by the Sandra's research group [4]. The low recoveries obtained for very hydrophobic compounds when the sample was magnetically stirred using conventional polytetrafluoroethylene (PTFE) bars are behind this proposal. This negative connotation was exploited by the authors to develop a very efficient microextraction technique, capable of competing with solid phase microextraction (SPME), a reference technique by past decade.

SBSE is based on the partitioning of the target compounds between the sample and the extracting phase immobilized in an inert support provided with a metallic element that allows the stirring of the unit. Among the main advantages of SBSE, the higher enrichment factors that are achieved can be cited. This is consequence of the larger amount of extractant that is used (as regards that used in SPME).

SBSE also features good reproducibility, high adsorption capacity and analyte recoveries and reduced organic solvent consumption. On the contrary, limited coating commercially available and the poor stability of laboratory-made units can be cited as main limitations.

### 2.1. Stir bar sorptive extraction modes

As it is the case with other extraction techniques, two operational procedures have been proposed for SBSE: direct immersion (DI) and headspace (HS) modalities. DI-SBSE involves the sorptive unit to be placed inside the liquid sample, being magnetically agitated for a fixed time at certain speed to complete the extraction. The HS modality is more scarcely used as it involves the stir bar to be placed in the HS of a vial containing the sample. The unit is then tightly closed and then the sample is either heated or stirred to facilitate the analytes transference to the gaseous phase and then to the coated bar. This modality is limited to the determination of volatile and/or thermally stable compounds, being also compatible with very acidic/alkaline, saline or dirty samples as the extraction unit remains separated from the sample during the whole extraction step.

Regardless the adsorption modality (DI or HS) selected, the desorption step can be implemented via liquid phase (liquid desorption) or by heating the bar (thermal desorption). The first is more appropriate in those cases where the analytes are retained by chemical interactions (electrostatic or chelation) while the latter can be used to break very weak interactions such as the Van der Waals forces.

Multiextraction modes allow the simultaneous extraction of analytes with different polarity: dual SBSE [5, 6], dual-phase dual SBSE [7], multishot [8] and sequential [9, 10] modalities have been proposed. However, these extraction modes are not practical enough as they require large sample volumes and are characterized for being time consuming.

A new configuration recently published allows the off/on-site sampling by attaching the stir bar to a homemade miniature battery-operated portable electric stirred for the determination of PAHs in water samples [11].

### 2.2. Trends in stir bar sorptive extraction coatings

As previously described, classic SBSE is mainly focused on the extraction of nonpolar or weakly polar compounds because of the hydrophobic nature of PDMS (polydimethylsiloxane) coating, which has been for years, the only commercially available extracting phase. Intensive research has been conducted to overcome this limitation and in consequence to extend the applicability of SBSE to more polar analytes. These strategies are mainly toward the use of PDMS-modified coatings or the development of new extraction coatings. To date, only polyethylene glycol (PEG) and polyacrylate (PA)-based polar coatings have been marketed by Gerstel [12].

The first in-house SBSE coatings were based on PDMS synthesized by sol-gel reaction that enables the chemical immobilization of the coating into the surface of a glass stir bar. This chemical binding presents some advantages such as mild preparation conditions, simple operation and the good mechanical and chemical stability of the resulting coating. By use of sol gel technology, stir-bar based on PDMS and polyvinyl alcohol (PVA) [13], polyethylene glycol (PEG)-PVA [14], beta-cyclodextrin (β-CD) [15], β-CD-divinylbenzene (DVB) [16], PVA and carbowax (CW) [15], tetraethoxysilane (TEOS) [17] or cyanopropyltriethoxysilane (CNTPrTEOS) [18] has been prepared. Polymerization reaction is another way to prepare PDMS-based coating, and polypyrrole (PPY) [19] or activated carbon (ACB) [20] has been synthesized with this procedure. Inorganic coatings in SBSE (mainly based on carbon-based material) includes octadecyl ($C_{18}$) [21] or amino-modified multiwalled carbon nanotubes (MWCNTs) PDMS [22]. Hybrid coating-based stir bar such as TEOS-PDMS [17] or partially sulfonated polystyrene (PSP)-$TiO_2$ [23] have also been reported.

Monolithic based coating consists of a rigid macroporous structure that is usually synthesized by in-situ polymerization of the monomer, cross-linker, initiator and the porogen. Monolithic materials are very versatile as the monomers determine the interaction chemistry (polar, nonpolar or mix modes) and the porogen solvent defines the porosity and

the mechanical stability of the coating. The preparation of these monolithic coatings is simple, relatively inexpensive and although are laboratory-made, their synthesis is highly reproducible. The introduction of monolithic material into SBSE coatings was proposed by Huang and Yuan [24]. Since then, many approaches have exploited the inherent properties of monolithic materials for the extraction of analytes covering a wide range of polarity [25]. Polyurethane foam is another versatile material that has been successfully employed as SBSE coating [26, 27] to task-specific analytical problems.

The use of molecularly imprinted polymers (MIPs)-coated stir bar was proposed for the first time by Zhu *et al.* [28]. MIPs are polymeric materials characterized for selectivity enhancement as they present very specific binding sites to recognized target analytes. The mechanical stability of the MIP coating is limited, and losses of coating during the extraction may occur as a consequence of the contact with the bottom of the vessel. To overcome this problem, Hu *et al.* proposed a dumbbell-shaped stir bar format for MIP-based extraction [29]. When dealing with biological samples, proteins usually clog the sorbent and a pretreatment step in which the proteins are precipitated is required. The sample preparation time is minimized using a biocompatible adsorbent such as restricted access materials (RAMs) [30, 31]. RAM-based coatings are employed to limit the interaction of the matrix with the active sites of the sorbent using a size-exclusion mechanism.

### 2.3. Novel stir bar sorptive extraction-based devices

One of the main limitations associated to laboratory-made SBSE devices is the detachment of the coating as a consequence of the continuous friction with the bottom vessel. A successful alternative is the so-called monolithic [32], PDMS [33] or silicone [34] rod/tube sorptive extraction. In this case, commercially available PDMS or silicone materials in the form of rods or tubes are used for the isolation and preconcentration of target compounds. In comparison with SBSE, there are advantages in terms of cost and robustness although the enrichment factors are similar to those provided by SBSE for the same analytes. Among the extraction modalities, they can work under DI and HS conditions [35]. Although a

stainless steel wire can be introduced inside the rod or the tube (i.e. stirred unit), the most accepted procedure involves sample shaken in both direct immersion and headspace modalities.

Another possibility to avoid coating damage is to use a porous material [i.e. hollow fiber (HF)] filled with the sorbent material. This modality, named carbon nanotubes assisted pseudo-stir bar solid/liquid microextraction, was proposed by Es'haghi *et al.* [36] in 2011 as an efficient alternative to determine brilliant green in fish pond water. They used a polypropylene HF membrane (1 cm in length) filled with 3 µL of MWCNTs dispersed in 1-octanol (acceptor phase). Then, the fiber was sealed at both ends by magnetic stoppers in such a way that it operates as a pseudo-stir bar. For elution, the stoppers are removed and the HF containing the acceptor phase enhanced with the analyte (brillian green) was transferred to a vial containing 2 mL of methanol for elution.

In the same way, dual solvent-stir bars microextraction [37] was proposed by Yu *et al.* The extraction unit is based on a stainless-steel stir bar with two HFs (containing the organic solvent) attached. The device, which is stirred by itself during extraction, was compared in terms of analytical performance to conventional U-shape HF liquid phase microextraction (LPME) for the determination of Sudan dyes from food samples. Later, a slightly modified configuration was employed for the determination of anabolic steroids in biological matrices [38]. In this approach, the HF is fixed to a stirring bar pedestal. As well as this, HF-based stirring extraction bars working under the liquid-liquid microextraction principles were also employed for the determination of hormones in milk [39] and cosmetic products [40]. In those works, however, the stirring extraction bars were prepared by inserting the stainless-steel into the HFs and once the extraction is performed, they are removed from the solution by means of a magnet.

## 3. DISK-BASED DEVICES

### 3.1. Rotating disk sorptive extraction

Rotating disk sorptive extraction (RDSE) was proposed by Richter *et al.* in 2009 [41] as an alternative to conventional SBSE that avoids the direct contact between the extracting phase and the vessel walls. As a consequence, higher stirring rates can be employed which enhances the analyte transference from the bulk solution to the extracting phase and reduces the extraction times. The RDSE unit, which is depicted in **Figure 1A**, consists of three main elements. In short, a PTFE disk acts as an inert support where a magnetic bar is embedded to allow the stirring of the device while a thin film of PDMS, the extracting phase, is deposited on one of the disk sides. The typical extraction procedure involves the introduction of the unit into the sample where it is stirred during a defined time in order to isolate the target analytes. After the extraction, the unit is taken out from the sample and immersed in an appropriate organic solvent for the chemical elution. Although the first prototype was evaluated for the extraction of nonylphenol from water samples, RDSE has shown great versatility on the resolution of different analytical problems. In this sense, RDSE has been applied to the determination of pesticides in river samples [42] and triclosan and methyltriclosan in water [43] by gas chromatography-mass spectrometry (GC/MS).

The chemical elution of the disk involves a dilution of the analytes before their instrumental determination that can be avoided if they are directly monitored on the disk surface by spectroscopic techniques. In this case, after the extraction, the thin PDMS film is detached from the PTFE disk and placed in a dedicated holder that allows the insertion of the film into the path light. Spectrophotometry in combination with RDSE has been employed for the determination of colored organic compounds such as malachite green [44] and crystal violet [45] in water samples. In addition, it has been applied to the determination of copper in water after its reaction with sodium diethyldithiocarbamate that forms a hydrophobic (extractable) and colored (measurable) complex [46]. In the in-film detection format, the

extraction temperature seems to be crucial as it enhances the analytes transference from the bulk sample to the PDMS film. In fact, the extraction temperature in the in-film detection format is in the range from 75 to 90ºC while the conventional approach (extraction/elution) is performed at room temperature.

As it is the case with SBSE, the use of PDMS phases limits the applicability of RDSE to the extraction of very hydrophobic compounds and therefore the use of other phases is desirable. Cañas and Richter proposed in 2012 the use of Empore$^{TM}$ octadecyl disks as sorptive phase in RDSE [47] for the extraction of hexachlorobenzene from water. The sorptive medium, which consists of membranes with embedded SPE microparticles, present high sorption capacity and its industrial production ensures a batch-to-batch reproducibility. The assembly of the membrane to the RDSE device is easily accomplished using silicone as organic binder. This binder does not affect to the extraction of the analytes as its excess is removed with dichloromethane and this attachment is stable enough to allow six consecutive extraction procedures without the membrane detachment. The new device shows a similar performance to that of conventional PDMS film. This fact indicates that the transference of the analytes from the bulk solution to the film is the rate limiting factor. Cañas *et al.* have recently proposed a new RDSE format that incorporates conventional SPE sorbents [48]. In this case, the classic RDSE device is modified including a cavity, as it can be observed in **Figure 1B**, where the sorbent is loaded. A fiber glass filter is attached to the device by means of an o-ring that avoids losses of the sorbent during the extraction. In the first application, Oasis HLB (hydrophilic-lipophilic balanced) sorbent, a copolymer of DVB and N-vinylpyrrolidone, has been employed for the extraction of the veterinary drug florfenicol from porcine plasma. The extraction procedure comprises the typical steps of a SPE protocol, namely: conditioning, extraction, clean up and elution, all of them being performed in the stirring devices that enhance the mass transference processes. Owing to the great variety of available SPE sorbents and their easy loading on the unit, this RDSE approach is highly versatile and useful. Another application of this modality is the determination of nonsteroidal anti-inflammatory drugs (NSAIDs) from waste water [49].

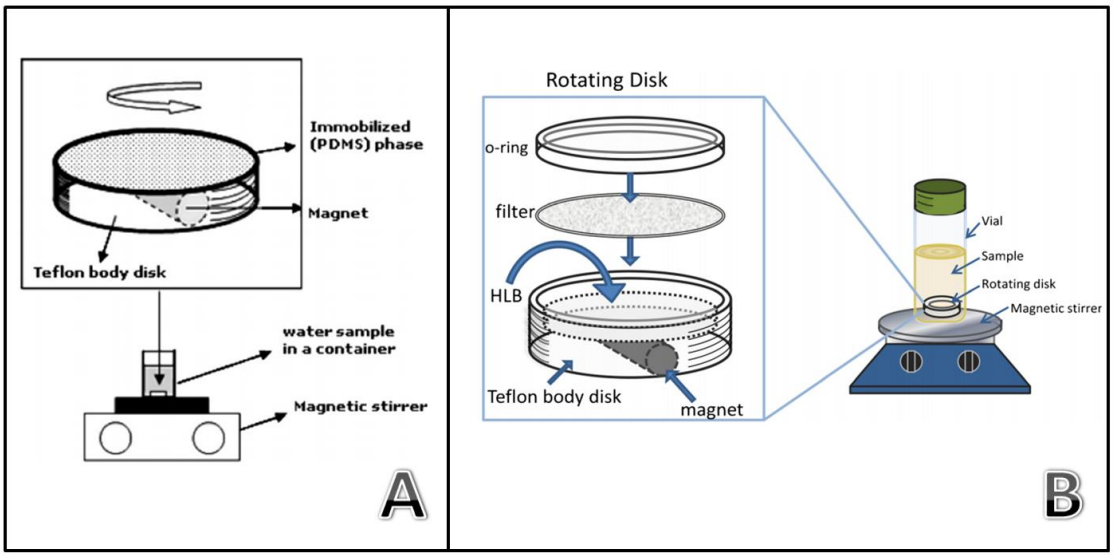


**Figure 1.** Schematic diagram of two rotating disk sorptive extraction (RDSE) devices. (**A**) Classic RDSE unit comprising a PDMS film as extractant phase, reproduced with permission of Elsevier from reference 43. (**B**) RSDE unit that incorporates conventional SPE sorbents as extractant phase. (Reproduced with permission from Ref. 41 and 48 © Springer, 2014).

The innovation of RDSE does not only comprise the development/use of new phases but also involve methodological advances. In a recent publication, Richter´s research group has evaluated two types of stirring, perpendicular and parallel, on the extraction kinetics [50]. Perpendicular stirring corresponds to classic RDSE where the extraction is developed in a common magnetic stirrer, the axis of rotation of the device being perpendicular to the sorptive film while parallel stirring involves the attachment of the unit to a rotary rod. The stirring mode has a clear effect on the kinetics and the parallel configuration reduces the equilibrium time from 80-100 min to just 30 min.

The potential of stirred disks in the microextraction context has been also evaluated by our research group. Stir frit microextraction is based on the use of a commercial polyethylene frit, from commercial SPE cartridges, as sorbent for the extraction of volatile compounds

from water samples [51]. A metallic wire is introduced into the frit to allow its stirring during the extraction, further chemical functionalization not being necessary. The extraction procedure consists of the introduction of the frit into the sample and its stirring during a defined period of time. Once the extraction is performed, the frit is withdrawn from the extraction vessel and introduced in an HS vial for the final GC/MS analysis. The analytes are thermally desorbed from the frit avoiding the chemical elution and therefore stir frit microextraction can be considered almost (55 µL of methanol are used for the frit conditioning) a solventless technique.

Borosilicate disks are also excellent supports for disk-based microextraction techniques due to their mechanical stability and porous structure. However, these supports should be derivatized to immobilize appropriate functional groups on their surface as bare disks do not present intense interactions with organic analytes. The derivatization requires a previous activation of the disk, which is performed by their immersion in a sulfuric acid/hydrogen peroxide (2:1, v/v) solution for 20 min at 100 ºC, to induce the in-surface generation of active hydroxyl groups that are further covalently modified. In this context, borosilicate disks modified in-surface with octadecyl groups have been proposed for the microextraction of triazine herbicides from environmental waters [52]. This approach works under the LPME mode as the hydrophobic chains of the modified disks are used to retain an organic solvent, toluene, which is the responsible for the extraction of the analytes. The disk is adapted to a dedicated device that allows its stirring into a large volume of sample (500 mL). High preconcentration factors, in the range from 79 to 839, are obtained and the limits of detection resulted to be in the low microgram per liter level. Borosilicate disks can be also modified with nanoparticles. In this sense, single-walled carbon nanohorns have been immobilized in the disk surface [53] to make possible the extraction of benzophenone-3 from swimming pool waters. Once synthesized, the SWNHs-disk (**Figure 2A**) is pierced with a screw and adapted to a rotating metallic axle that can be integrated in a portable drill (**Figure 2B**) that makes feasible the stirring of the unit into the sample (**Figure 2C**). The extraction device is quite simple and is potentially portable, which opens a

door to on-site extractions. The enrichment factor and extraction recovery, which are 1379 and 68.9% respectively, reveal the great potential of the technique.

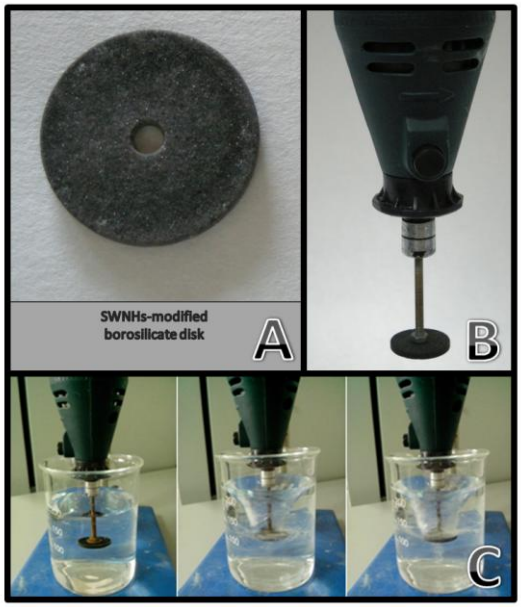


**Figure 2. (A)** Borosilicate disk with single-walled carbon nanohorns immobilized in its surface. (**B**) Integration of the SWNHs-disk to a portable drill by means of a rotating metallic axle. (**C**) Typical extraction procedure.

### 3.2. Stir-cake sorptive extraction

Stir-cake sorptive extraction (SCSE) was proposed in 2011 as a novel technique that enables the use of monolithic sorptive phases in stirring devices [54]. As it was the case with RDSE, SCSE improves the mechanical stability of these polymeric extraction phases avoiding their direct contact with the vessel walls thanks to the use of a dedicated device. The core of the device, which is presented in **Figure 3**, is a plastic holder obtained from a conventional SPE cartridge. The plastic holder is pierced with a glass protected iron wire in order to make the

device's stirring feasible while a monolithic disk with defined dimensions and chemical properties is located in the upper part of the unit.

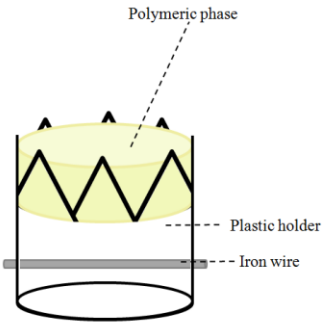


**Figure 3.** Schematic diagram of the stir cake sorptive extraction device comprising a monolithic phase as extractant.

In the conventional extraction protocol, the device is introduced into a large volume of sample and stirred for an appropriate time at a fixed rate in order to isolate the analytes form the bulk sample. After the extraction, the unit is withdrawn from the solution and immersed in a low volume of an organic solvent to elute the analytes for their further instrumental determination.

The initial study of SCSE showed that several variables may affect the extraction efficiency of the technique, the thickness and diameter of the sorptive phases playing a key role. In this sense, the diameter of the disk has a clear and positive influence on the sorption capacity but it also increases the volume of organic solvent required for the elution. The thickness of the disk has a similar positive effect but it affects negatively the extraction kinetics. The first prototype of SCSE used a monolithic of poly(vinylimidazole)divinylbenzene as sorptive phase for the extraction of steroids hormones from milk samples. This initial study showed acceptable absolute extraction recoveries even for this complex analytical problem.

The research performed in SCSE in the past years has been focused on the use of different sorptive phases to increase the applicability of the technique. The SCSE inventors proposed in 2012 the use of a polymeric ionic liquid (PIL) monolithic phase for the extraction of inorganic anions, such as: $F^-$, $Cl^-$, $NO_2^-$, $Br^-$, $NO_3^-$, $PO_4^{3-}$ and $SO_4^{2-}$ from water samples [55]. The PIL was synthesized by copolymerization of 1-alkyl-3-methylimidazolium chloride and ethylene dimethacrylate in a mixture of 1-propanol and dimethylformamide in the presence of azoisobutyronitrile. Chloride acts as counter ion in the polymeric network and therefore it is key in the anion exchange procedure that consists of several steps (all of them performed in the stirred unit). First of all, the PIL cake is conditioned in a sodium hydroxide solution where the initial chloride counter ion is substituted by hydroxyl anion. The conditioned cake is stirred into the solution to extract the target anions by the anion exchange mechanism, the analytes being finally recovered using a sodium hydroxide solution as eluent. The final extract is analyzed by ionic chromatography providing limits of detection as low as 0.11 µg/L with good precision levels.

PIL sorptive phases with balanced functionalities can be also employed for the simultaneous extraction of organic and inorganic compounds from water samples [56]. For this purpose, Huang *et al.* synthesized a PIL containing 1-vynylbenzyl-3-methylimidazolium chloride and DVB as anion exchanger and hydrophobic monomers, respectively. The resulting polymer presented an optimum porosity, with average macropores of 1100 nm and mesopores around 300 nm, which confers to the polymer a high superficial area (19.6 $m^2/g$). PILs phase synthesized from 1-allyl-3-methylimidazolium chloride and ethylene dimethacrylate has been also employed for the extraction of organic acid preservatives (namely sorbic acid, benzoic acid and cinnamic acid) from juices and soft drink samples [57]. In this case, the analytes are extracted by a double mechanism, anionic exchange and hydrophobic interactions, which improve the selectivity of the process. Recently, PILs have been also employed for the determination of benzimidazole anthelmintics in water, honey and milk samples [58].

The extraction of polar analytes is one of the limitations of conventional SBSE since the classic phase (PDMS) is essentially hydrophobic. Huang *et al.* [59] have recently proposed the use of poly(allyl thiourea-co-divinylbenzene) as polymeric phase in SCSE for the extraction of polar phenols. The extraction of others polar compounds (amines) has been accomplished with graphene oxide nanosheet incorporated in the monolithic SCSE material [60]. The determination of sulfonamides in water samples was performed with a new sorbent based on poly(4-vinylphenylboronic acid-divinylbenzene) (VPB-DB) monolith [61].

## 4. FLAT MEMBRANE-BASED DEVICES

### 4.1. Stir-membrane extraction

#### *4.1.1. Stir membrane extraction and related techniques under the solid-phase extraction and solid phase microextraction formats*

Stir membrane extraction (SME) was proposed in 2009 by Alcudia-León *et al.* [62] as a new sample preparation technique that combines in the same device the excellent extraction capabilities of flat polymeric membranes (PMs) and the well-known beneficial effect of stirring. The design of the extraction unit as well as the assembly process is schematically depicted in **Figure 4**. The laboratory-made device was constructed using four commercially available elements: (i) laboratory-made polyvinyl chloride (PVC) protected iron wire (1.4 cm long), (ii) an internal part which is cut from a commercial 3- mL SPE cartridge (1 cm internal diameter and 0.6 cm height), (iii) a PTFE membrane (2.5 cm diameter) and (iv) an external element that is obtained from a pipette tip (1.2 cm internal diameter and 0.5 cm height). The assembly process is very simple. The hydrophobic PTFE membrane is placed in the upper part of the internal cylinder, and the unit is sealed by displacing the external element though the internal one. It is important to highlight that the dimensions of both cylinders are critical as they should perfectly fit together in order to guarantee that the membrane is

tightly fixed. Then, an iron wire is introduced through pre-pierced orifices, what makes feasible its magnetic stirring.

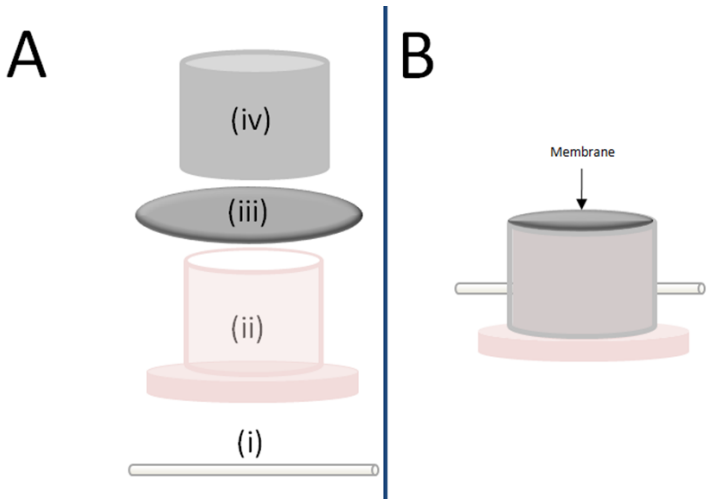


**Figure 4.** Diagram of the stir-membrane extraction device. (a) Main elements: (i) iron bar, (ii) upper part of a commercial solid-phase extraction cartridge, (iii) PTFE membrane, and (iv) section of a 5-mL pipette tip. (b) Final assembly of the unit. (Reproduced with permission from Ref. 3 © Springer, 2012).

SME works under the principles of SPE and the isolation of the analytes takes place when the extraction unit is introduced into the sample and stirred at a defined velocity for a fixed time. The extracted analytes are finally eluted for their analysis. As this technique presents evident similarity with SBSE, a critical comparison of both techniques was developed using polycyclic aromatic hydrocarbons (PAHs) as model analytes. In this sense the SME device, containing a PTFE membrane with a superficial area of 113 $mm^2$ in each face, was compared with three different PTFE stir bars (622, 433 and 89 $mm^2$ of superficial area). In all the cases, the stir membrane unit provided the best extraction results, which can be ascribed to the membrane porosity.

As it has been previously indicated, chemical elution involves a dilution of the analytes that may negatively affect the sensitivity levels. To avoid this limitation, Alcudia-León *et al.* proposed the direct coupling of SME with infrared spectroscopy for the determination of the hydrocarbon index [63], which allowed the direct monitoring of the analytes in the PM surface. In this case, the characteristics of the membrane are critical since it should present chemical affinity towards the analytes and it should present negligible absorption in the infrared region selected for analyte determination.

One of the main shortcomings of SME is the limited adsorption capacity of conventional membranes that restricts the volume of sample that can be processed. This limitation can be overcome changing the nature of the extracting phase. In this context, stir fabric phase sorptive extraction (SFPSE) has been recently proposed [64]. Fabric phases (FPs), developed by Kabir and Furton [65], are produced by sol-gel technology [66] giving rise to an inherently porous hybrid inorganic-organic sorbent material chemically bonded to the flexible and porous substrate matrix. The high mechanical stability of FPs and the different chemistries available (polymeric coating of diverse polarity, carbon nanoparticles embedded phases etc.) give this material a great potential. FP material has been used as extractive phase in a microextraction procedure for the determination of estrogens in urine samples [67]. In this approach, a teflon-coated magnetic stirring bar was employed whereas the FPs was floating in the sample. Owing to their planar structure, FPs can be easily adapted to the conventional SME device. The first prototype of SFPSE has been initially evaluated for the determination of three classes of environmental pollutants including triazine herbicides, PAHs and chlorophenols (CPs). For this purpose, two flexible fabric substrates, cellulose and polyester were used as the host matrix for the three different sorbents: sol-gel polytetrahydrofuran (PTHF), sol-gel polyethylene glycol (PEG) and sol-gel polydimethyldiphenylsiloxane (PDMDPS). Due to the inherent porosity of sol-gel derived extraction sorbent and the built-in pores of the host substrates, fabric phase sorptive extraction (FPSE) medium has shown high analyte enrichment capacity in a relatively short period of time. In addition, the wide range of sol gel-based sorbents available with tunable

selectivity and porosity made SFPSE a high promising technique for environmental, pharmaceutical, toxicological, clinical and forensic application.

Alcudia-León *et al.* have also proposed a modification of conventional SME to enhance the extraction capacity in the so-called magnetically confined hydrophobic nanoparticles extraction [68]. This approach, which works under the SPE principles, is based on a dedicated device that is depicted in **Figure 5**. The unit shares some elements with the classic stir membrane extraction device although the core elements are completely different. In this configuration, hydrophobic magnetic nanoparticles (MNPs) are used as extractant phase and are deposited in the unit forming a thin extracting layer that presents an optimal surface-to-volume ratio. The layer is stabilized by a small cube magnet that also makes feasible the magnetic stirring of the extraction unit. This extraction technique takes advantage of the great extraction capabilities of hydrophobic MNPs and it gives better results than the conventional magnetic SPE procedure that consists of the dispersion of the sorbent into the sample. This is due to the high aggregation tendency of these MNPs which makes their dispersion impracticable.

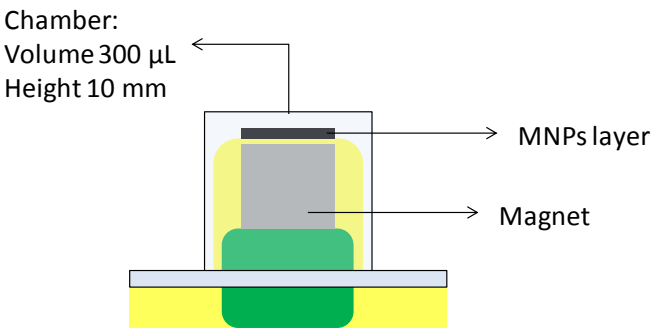


**Figure 5.** Diagram of the device employed in the so-called magnetically confined hydrophobic nanoparticles microextraction. (Reproduced with permission Ref. 68 © Springer, 2012).

### *4.1.2. Stir membrane extraction under the liquid phase microextraction (LPME) format*

The versatility of SME has also been exploited in the LPME format bringing on the development of stir membrane liquid phase microextraction (SM-LPME) [69]. SM-LPME tried to improve the extraction capacity of SME using a solvent instead of a membrane, as extracting phase. The original approach was based on two-phase mode that is especially applicable for the extraction of non-polar compounds from aqueous matrix. In this case, the extraction device is modified in order to create a chamber (about 50 µL) where the extraction solvent (acceptor phase) is located. The design of the extraction device is shown in **Figure 6**. In this approach, the organic solvent should fulfill some requirements. First of all, it must have good affinity for the target analytes in order to isolate them from the sample matrix. Moreover, it should be chemically compatible with the membrane employed and it should have a low solubility in water to prevent losses during the extraction. Finally, the organic solvent should be compatible with the instrumental technique.

SM-LPME was firstly evaluated for the isolation and preconcentration of CPs from water samples using a ethyl acetate:toluene (1:1, v/v) mixture as extractant, containing N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) as derivatization reagent. BSTFA improves the GC analysis of the analytes and at the same time increases their extraction making them more hydrophobic.

The main drawback of SM-LPME is the stabilization of the solvent in the extraction unit that limits the extraction time and specially the sample volume that can be processed. A three-phase LPME format can overcome this problem as a thin film of an appropriate organic solvent stabilized in the pores of the membrane is used as supported liquid membrane (SLM) between two aqueous media (the sample and the acceptor phase). This format presents a great potential for the extraction of basic or acidic analytes (ionisable nonpolar compounds which present a pH-dependent electrostatic charge) from aqueous samples. Stir membrane liquid-liquid-liquid microextraction (SM-LLLME) was proposed in this

context for the first time to isolate and preconcentrate 11 phenols from water samples prior to their determination by HPLC-UV giving detection limits in the nanogram per liter range [70].

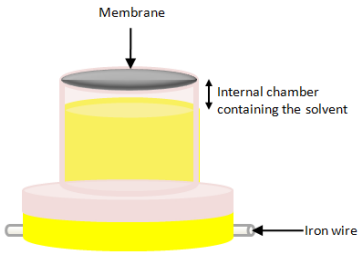


**Figure 6**. Scheme of the device employed in stir membrane liquid-liquid extraction.

SM-LLLME improves the extraction selectivity and therefore it is specially interesting for bioanalysis. Two reasons support this selectivity enhancement. On the one hand, the pH gradient is selected taking into account the pKa values of the target analytes. On the other hand, the SLM acts as physical barrier avoiding the transference of macromolecules, such as proteins, from the sample to the aqueous acceptor. The potential of SM-LLLME in bioanalysis was evaluated by the determination of NSAIDs in urine samples [71]. The combination of SM-LLLME and HPLC-UV allowed the determination of these compounds at therapeutic doses with good recovery and precision levels.

The conventional SM-LLLME device cannot process sample volumes lower than 10 mL. This is a main shortcoming in bioanalysis especially when samples of limited volume (e.g. saliva, sweat or plasma) are considered. The adaptation of the SM-LLLME to the extraction of volume-limited biological specimens has also been recently published [72]. The proposal was evaluated using the determination of paracetamol in saliva samples as model analytical problem. Saliva sample is quite interesting due to its noninvasive, rapid, economical and

easy sampling. Moreover, for many drugs and biomolecules there is an excellent correlation between saliva and blood concentration. For this reason, saliva analysis is gaining interest to obtain bioanalytical information. In the new configuration of stir membrane device, the height of the external element is increased up to 4 cm generating an upper chamber where the sample is located (**Figure 7**). In this way, the sample is integrated in the extraction device allowing processing lower sample volumes.

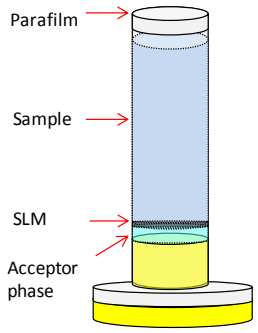


**Figure 7**. Adaption of the stir membrane liquid-liquid-liquid extraction device to process low volume of sample. SLM, supported liquid membrane.

The versatility of SME devices allows their use for the treatment of solid samples. This novel approach, which is called stir-membrane solid-liquid-liquid microextraction (SM-SLLME) [73], has been evaluated for the determination of parabens in lyophilized human milk samples. As breast milk is the main route of exposure for breastfed infants, the analysis of these endocrine disrupting compounds is of special interest. To date, current sample preparation of breast milk is tedious and typically involves a previous acid treatment and a centrifugation step to release the target analytes from the sample matrix. However, these steps are avoided with this new methodology. In this case, the extraction setup has been simplified and comprises two simultaneous extractions that take place in the same device. First of all, the analytes are solid-liquid extracted with an appropriate solvent and in a

second step a LLE between the organic solvent and an aqueous acceptor phase at the appropriate pH is performed. The integration of both extractions allows not only the improving of basic analytical properties but also the simplification and miniaturization of the sample treatment. The critical step in this procedure is the selection of the organic solvent, which should fulfill some requirements. On the one hand, it should be able to break the analyte-matrix interaction because the compounds present a marked interaction with the fatty compounds of the sample. On the other hand, the solubility of the analytes in the aqueous acceptor phase should be higher than in the organic donor phase

For the development of the SM-SLLME procedure, a special unit is required. The unit, which is depicted in **Figure 8**, consists of only two commercial elements: an Eppendorf tube of 2 mL and a hydrophobic PTFE membrane. In the assembly process, the Eppendorf is filled with the solid sample and an appropriate organic solvent while the cap of the Eppendorf is filled with the acceptor phase. The PTFE membrane separated the two chambers and it was fixed by press-fit (squeezed) closing the Eppendorf. The extraction device is agitated in an eight-position digital agitator-vibrator allowing the solid-liquid extraction of the PBs from the solid sample to an organic media and the subsequent liquid-liquid extraction of the analytes from the organic media to an alkaline aqueous acceptor phase. After SM-SLLME, the aqueous acceptor solutions were analyzed directly with ultra performance LC-MS/MS. The new proposal seems to be versatile enough to face up the isolation and preconcentration of hydrophobic ionizable compounds from different solid matrices.

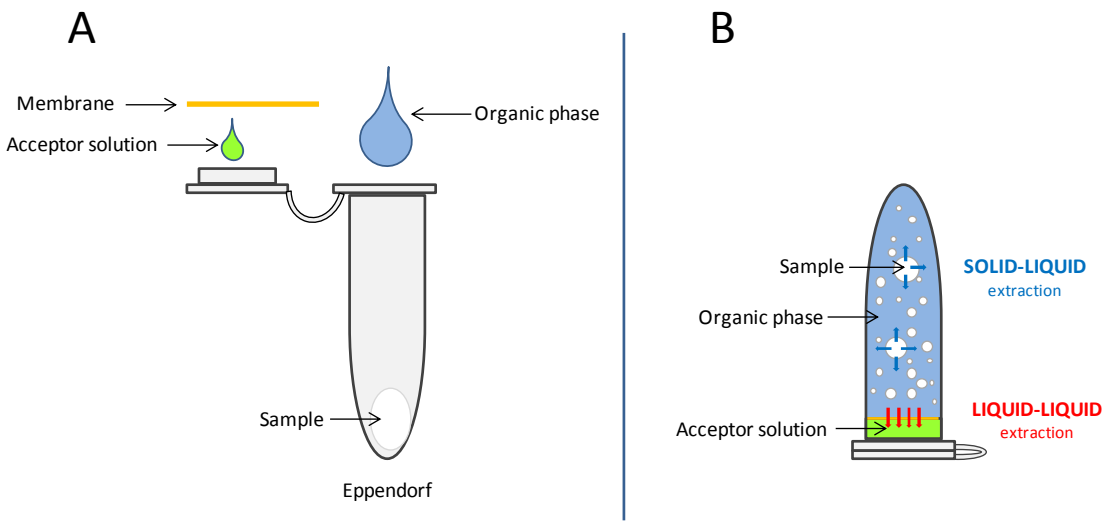


**Figure 8**. Diagram of the (a) assembly and (b) final state of the device employed in SM- SLLME.

### 4.2. Parallel artificial liquid membrane extraction

PMs have also been recently employed by Pedersen-Bjergaard *et al.* in a new approach termed parallel artificial liquid membrane extraction (PALME) [74]. PALME is a miniaturized liquid phase extraction method performed in a multiwell plate format. The experimental setup employed in PALME (**Figure 9**) is based on (i) 96-well plate with 0.5 mL wells (donor plate) (ii) 96-well plate (acceptor plate) with polypropylene membrane (100 µm thickness) and (iii) a lid to avoid potential losses of the acceptor solution by evaporation. In the assembly process, the donor plate and acceptor plate create a sandwich in which each sample and acceptor solution is separated by the SLM. A lid was located above the acceptor plate to avoid partial evaporation. PALME could perform 96 samples simultaneously in 15-60 min. After that, the aqueous acceptor solutions were analyzed directly with LC.

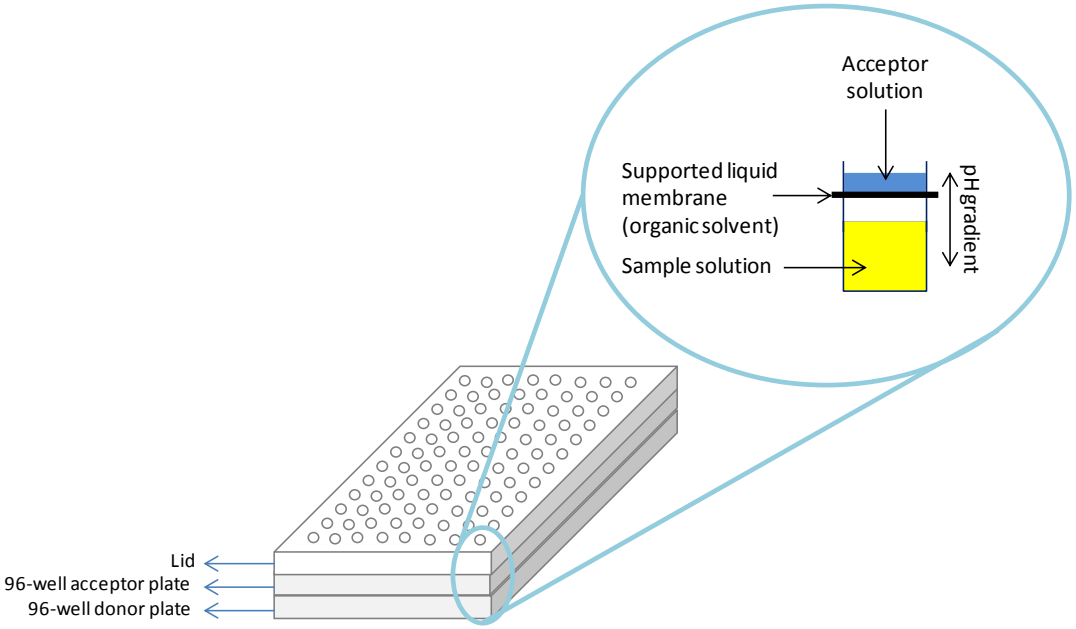


**Figure 9**. Scheme of the experimental setup employed in PALME.

PALME works under the three-phase LPME mode, which is specially appropriate for the extraction of ionizable hydrophobic drugs, their extraction being pH dependent. As in any three-phase mode, during the extraction in the sandwich-like system, analytes were extracted as neutral species from the sample small volume of biological sample, through the flat membrane impregnated with organic solvent into the aqueous acceptor phase with the pH adjusted. The existence of this double equilibrium provides high selectivity since only ionic hydrophobic analytes are extracted while nonionizable material remains in the organic solvent [75]. The proposed methodology has shown to be an effective extraction method for pethidine, nortriptyline, methadone and haloperidol (basic drugs) [74] and NSAIDs (acid compounds) [76] from plasma.

The authors also studied the influence of the membrane composition. Initial experiments were carried out using polyvinylidene fluoride (PVDF) membranes; however, nonspecific binding of the drug substances to the PVDF membrane occur and thus this membrane substituted by a PP membrane. The organic solvent (2 µL of dihexylether) employed to form the SLM rapidly permeated into the pores of the PP membrane and it is immobilized by capillary forces.

### 4.3. Electromembrane extraction

Electromembrane extraction (EME) was introduced in 2006 by Perdersen-Bjergaard *et al.* [77] with the aim to overcome the relatively slow extraction speed in HF-LPME. Up to date, most EME applications have been performed with HFs as mechanical support for the supported liquid membrane (SLM) [78]. However, other configurations have also been suggested in the past five years such as drop-to-drop EME based on a flat membrane [79] or micro-fluidic chip EME [80].

EME approach which combines extraction and stirring in the same device is based on flat membranes [81]. The laboratory-made extraction system, has been constructed using four commercial elements: (i) a piece of a 10-1000 µL pipette tip with the narrow end cut off (ii) a flat PP membrane (100 µm) (iii) a 2.0 mL Eppendorf and (iv) two platinum wires with "L-shape".

The first step in the assembly process is to solder the flat membrane to the wide end of the pipette tip. These two elements define the acceptor compartment (about 600 µL) that was filled with the extraction solution once the water-immiscible organic solvent is immobilized in the porous of the membrane. Later on, the Eppendorf was filled with the sample solution (about 600 µL). The pH conditions in the sample and in the acceptor phase should ensure efficient ionization of the analytes. The acceptor compartment is inserted into the sample compartment with a gap of approximately 1 mm. For cations, the "L-shape" anode and cathode (platinum wires) were placed oppositely into the sample and acceptor solution,

respectively. A voltage of 250 V was applied to initialize the extraction process, meanwhile the whole setup was agitated at a define velocity for a fixed time. As a general rule, the optimal magnitude of the potential difference usually varies from 9 to 300 V because higher voltages could probably lead to electrolysis or bubble formation among other negative effects. This electrical field is the driving force of the electrokinetic migration and its magnitude and direction of this is easily controlled by the power supply.

In classic EME, the volume of acceptor phase (typically 50-100 µL) was limited to the internal volume of the lumen of the hollow fiber. With this new EME set-up, the volume of acceptor phase is increased up to 600 µL and consequently the extraction recoveries increase as the amount of the target analytes trapped in the SLM is reduced. Flat membrane-based device for EME was applied for the determination of quetiapine, citalopram, amitriptyline, methadone and sertraline from human plasma with RSD values lower than 10% and extraction recoveries in the range from 83% to 105%. Although numerous benefits have been reported with EME, the sample throughput (number of samples that can be processed simultaneously) has been limited and considered as a drawback compared to other extraction techniques which have been incorporated into platform. With the aim to overcome this limitation, the idea of parallel electromembrane extraction (Pa-EME) was briefly investigated in 2010 with the hollow fiber configuration in which three extractions unit were connected in parallel to a 9 V battery as power supply [82]. However, Pa-EME with hollow fiber was challenging to operate. For this reason, Pa-EME with flat membranes configuration was developed in 2014 by the research group of Prof. Pedersen-Bjergaard [83]. In this setup, amitriptyline, fluoxetine, quetiapine and sertraline were isolated into formic acid and eight plasma samples were processed simultaneously in separated wells within 8 min of extraction with RSD values in the range 5-15%. In this configuration, the principles are the same as described previously, but two small modifications in the extraction device were introduced to make feasible the extraction of eight samples in parallel. On the one hand, the membrane was sealed to strips of eight plastic vials instead of a pipette tip. On the other hand, sheets of aluminum foil

were fixed with glue to the acceptor and donor well to serve as electrodes and connected to a power supply. The Pa-EME is placed on a shaker platform and subject to a given agitation rate throughout the extraction.

Very recently, the approach of Pa-EME with flat membranes take a big step forward with the 96-well format [84] (see **Figure 10**) increasing the total number of samples processed in parallel from 8 to 96. For this purpose, two 96-well collection plates were employed as donor and acceptor solution compartment. Moreover, different biological matrices were investigated to assess if the sample composition influenced the extraction performance and they were found to be independent, which increase the interest of EME in the bioanalytical field to solve existing and novel analytical challenges.

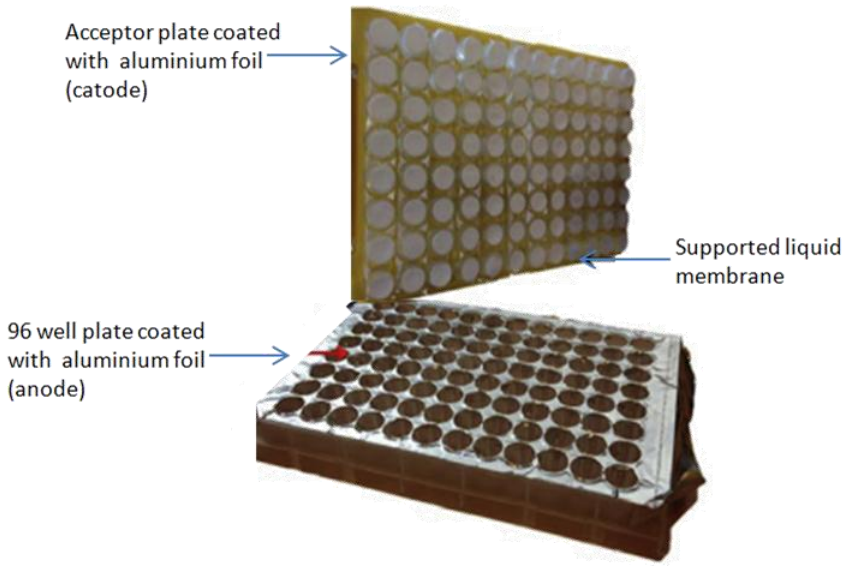


**Figure 10.** Lab-made device for the development of EME that integrates extraction and stirring. Reproduced with permission from Ref. 84 © Elsevier, 2014.

## 5. CONCLUDING REMARKS

Microextraction techniques with the stirring integrated in the same device have been in continuous evolution since the development of SBSE. Innovations in this field follow two different trends. On the one hand, efforts are mainly toward the development of new extraction coatings (i.e. sol-gel technology or monolithic materials) with the aim to extend the versatility and in consequence promoting the extraction of more polar compounds. The other research line is related to the development of new configurations of the extraction/stirring devices to avoid some inherent problems of the SBSE such as the mechanical instability of some coatings. The main sample preparation techniques in this context have been reviewed.

Nevertheless, automation is the bottle neck of these techniques that limits its application in routine analysis. In recent years, new emerging extraction/stirring approaches in the multiwell format under the SPME (i.e. 96-blade thin-film) have been successfully integrated in existing automation robots. This concept has been partially introduced in liquid-phase microextraction devices (i.e. PALME and Pa-EME), which may developed into a fully automated sample preparation techniques in the near future.

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BLOQUE II

# HERRAMIENTAS ANALÍTICAS

En el desarrollo experimental de la presente Tesis Doctoral se han empleado diferentes herramientas analíticas, que se describen en este Bloque. Tal es el caso de analitos, reactivos y otros materiales, muestras, la instrumentación empleada y aparatos. Por último, se describen los sistemas de microextracción con agitación integrada propuestos.

## II.1. ANALITOS, REACTIVOS Y OTROS MATERIALES

Durante el desarrollo de la Tesis Doctoral se han utilizado los siguientes reactivos:

**II.1.1. Analitos.** Los compuestos objeto de estudio se enumeran a continuación agrupados por familias:

- **Anti-inflamatorios.** Son un grupo de fármacos que actúan bloqueando la síntesis de prostaglandinas y su efecto es anti-inflamatorio (disminuye la inflamación), analgésico (disminuye el dolor) y antipirético (baja la fiebre). Se han utilizado ketoprofeno, fenoprofeno, diclofenaco, flurbiprofeno, ibuprofeno y gemfibrozil, de Sigma Aldrich. Las estructuras se muestran en la **Figura 1**.

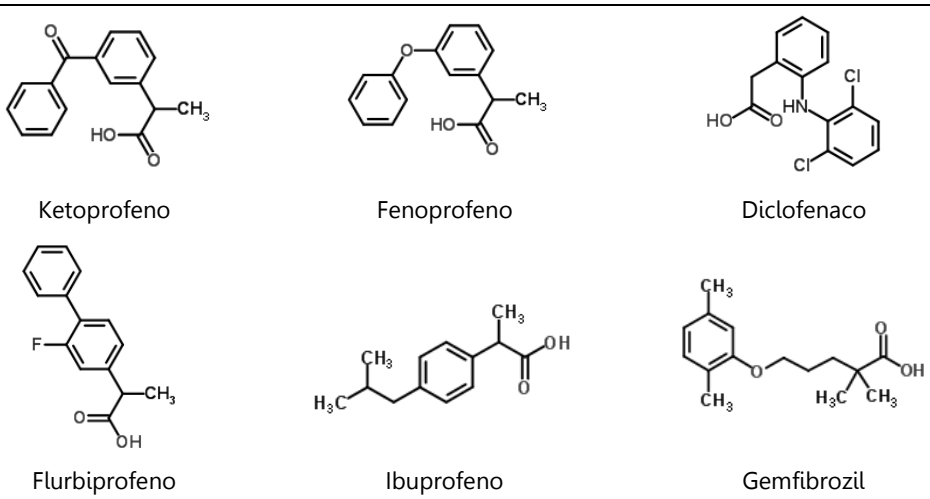


**Figura 1.** Estructura química de los anti-inflamatorios.

- **Benzofenonas**. Son cetonas aromáticas que se utilizan principalmente como componentes en productos cosméticos y en protectores solares. Estos compuestos atenúan el efecto nocivo de los rayos ultravioleta sobre piel y pelo, protegiéndolos. La benzofenona-3, también conocida como oxibenzofenona, es la más utilizada. Este grupo de compuestos ha sido recientemente clasificado como disruptores endocrinos (disruptores hormonales), ya que son sustancias que alteran el normal funcionamiento de procesos fisiológicos. Se han empleado benzofenona-1, benzofenona-3, benzofenona-8 y 4-hidroxi-benzofenona, suministradas por Sigma-Aldrich. En la **Figura 2** se muestran las estructuras de los compuestos estudiados.

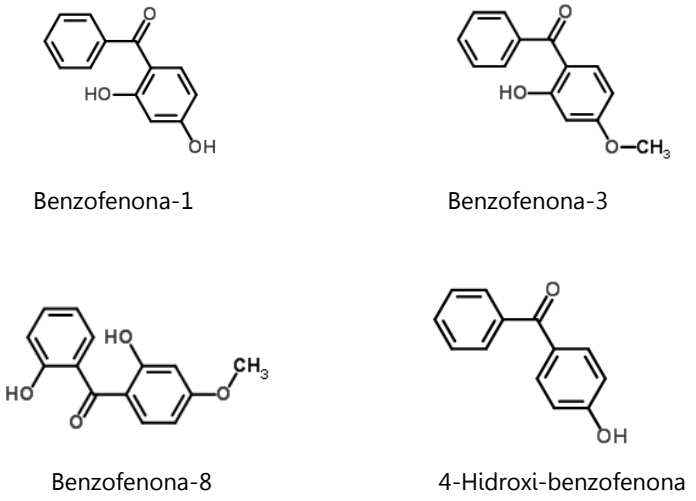


**Figura 2.** Estructura química de las benzofenonas.

- **Compuestos volátiles monoaromáticos**. En este grupo de analitos se incluyen el benceno, tolueno, etilbenceno, los isómeros orto-, meta- y para- xileno, y estireno. El *o*-xileno-$d_{10}$ se empleó como estándar interno. Todos se adquirieron en Sigma-Aldrich. Estos compuestos aparecen principalmente en el petróleo y sus derivados como la gasolina. Se encuentran como contaminantes en los distintos

compartimentos ambientales, principalmente en aquellos lugares que están cercanos al almacenamiento de carburantes. Las estructuras se muestran en la **Figura 3**.

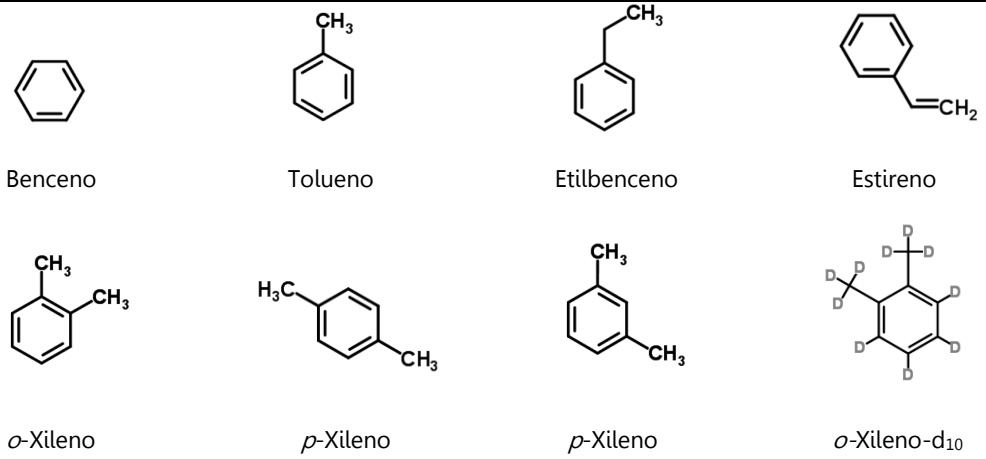


**Figura 3.** Estructura química de los compuestos volátiles monoaromáticos.

- **Paracetamol** (acetaminofen), de Sigma-Aldrich. Se trata de un fármaco con propiedades analgésicas que se utiliza ampliamente para el tratamiento del resfriado común y la gripe. Su bajo precio y su amplia disponibilidad ha dado lugar a intoxicaciones por dosis elevadas. Su estructura se muestra en la **Figura 4**.

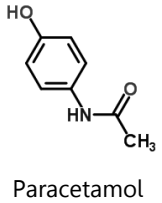


**Figura 4.** Estructura química del paracetamol.

- **Triazinas**. Las triazinas son compuestos cuya estructura consta de un anillo heterocíclico, similar a un anillo de benceno, pero con tres átomos de nitrógeno sustituyendo a tres átomos de carbono. Esto hace que existan tres isómeros de la triazina, en función del lugar que ocupen dichos átomos de nitrógeno. Se utilizaron simazina, simetrin, atrazina, secbumetón, prometón, terbumetón, propazina, prometrin y terbutrin, de Sigma-Aldrich. En la **Figura 5** se muestran las estructuras químicas de los herbicidas de triazinas empleados.

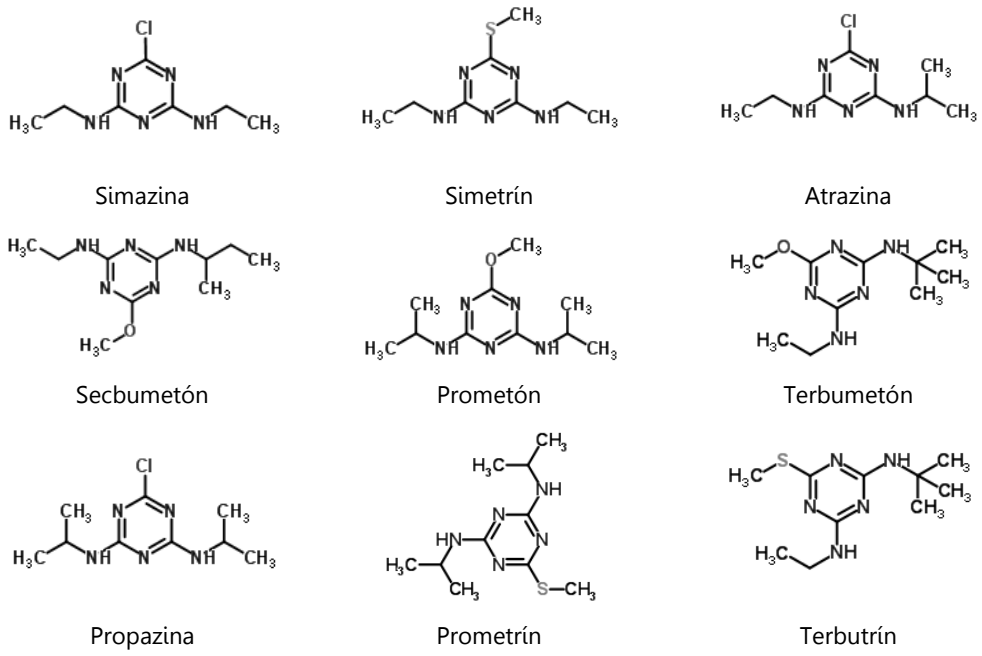


**Figura 5.** Estructura química de las triazinas.

## II.1.2. Reactivos

- Ácidos: ácido ortofosfórico (Panreac), ácido clorhídrico y ácido sulfúrico de Sigma-Aldrich.

- Bases: hidróxido sódico de Sigma-Aldrich e hidróxido amónico de Merck.
- Sales: cloruro sódico, de Sigma-Aldrich.
- Otros reactivos: cloruro de polivinilo para la crear película protectora en las barras agitadoras. Trimetoxi(octadecil)silano, como agente derivatizante, para introducir grupos octadecilo. Peróxido de hidrógeno, 3-aminopropiltrietoxisilano (APTS), N-3(dimetilaminopropil)-N-etil-carbodiimida (EDC·HCl), glutaraldehido (GA), borato sódico, N,N'-dimetilformamida (DMF). Todos fueron adquiridos en Sigma-Aldrich.

### II.1.3. Disolventes orgánicos

Los disolventes orgánicos se han empleado con distintas finalidades: (i) para la preparación de estándares de los analitos; (ii) como extractantes y eluyentes; (iii) como membranas líquidas soportadas; (iv) para limpiar y acondicionar materiales sorbentes; y (v) como componentes de las fases móviles utilizadas en los sistemas cromatográficos.

- Metanol (grado HPLC de J.T. Baker y grado LC-MS de Sharlab).
- Acetonitrilo, etanol, tolueno, acetona anhidra, 1-octanol (Panreac).
- 2-hexil-1-decanol, nonanol, 2,2-dimetil-1propil benceno y dihexil eter (Sigma-Aldrich).
- Dodecil acetato y 2-nitrofenil octil eter de Fluka (Buchs, Suiza).
- Isopentil benceno de Industria Química de Tokio (Tokio, Japón).

### II.1.4. Sólidos sorbentes y membranas

- Fritas de polietileno (PE) de las que se utilizan habitualmente en los cartuchos de extracción en fase sólida de 3 mL, con tamaño de poro de 20 µm, de Análisis Vínicos. Estas fritas se emplearon para la extracción de los compuestos orgánicos volátiles en muestras de agua.
- Membranas empleadas en la separación de fases en las técnicas de microextracción líquido-líquido-líquido:

  - Membranas de politetrafluoroetileno (PTFE, teflón) con un espesor de 100 µm y tamaño de poro de 0.5 µm, (Miarco, Valencia, España).
  - Membranas de polipropileno (PP) con espesor de 100 µm y tamaño de poro de 0.1 µm (Accurel PP 1E R/P, Membrana, Wuppertal, Alemania).
  - Membranas de fluoruro de polivinilideno (PVDF) (MA, USA).
- Fases extractivas sintetizadas y proporcionadas por el Instituto Internacional de Investigación Forense del Departamento de Química y Bioquímica de la Universidad Internacional de Florida (Florida, USA). Para su síntesis, detallada en el **Capítulo 4**, se utilizaron:
  - Polímeros sol-gel: politetrahidrofurano, poli (dimetildifenilsiloxano) o polietilenglicol, de Sigma-Aldrich (St. Louis, Mo, USA), Gelest (Morrisville, PA, USA), AlfaAesar (Ward Hill, MA, USA), respectivamente.
  - Sustratos flexibles para las fases extractivas: celulosa o poliéster de Jo-Ann Fabric (Miami, FL, USA).
  - Distintos reactivos y disolventes: acetona, diclorometano, metiltrimetoxisilano, y ácido trifluoroacético, de Sigma-Aldrich (St. Louis, Mo, USA). Hidróxido sódico y ácido clorhídrico de Thermo Fisher Scientific (Milwaukee, WI, USA).
- Discos/filtros de borosilicato de la casa comercial ROBU (Glasfilter-Geraete GmbH, Alemania). Se utilizaron dos tipos de discos, unos con tamaño de poro de 10-16 µm y otros de 16-40 µm, ambos de 20 mm de diámetro.
- Nanopartículas, concretamente se utilizaron nanocuernos de carbono de pared simple (SWNHs). Estas nanopartículas fueron suministradas por Carbonium S.r.l. (Padova, Italia) con pureza del 90%, longitud de 40 a 50 nm con diámetro de 4 y 5 nm. Los nanocuernos de carbono de pared simple pueden ser considerados como nanoestructuras con forma cilíndrica (similar a los nanotubos de carbono) con una terminación cónica en uno de sus extremos. Dicha terminación cónica se debe a la presencia de 5 pentágonos en el vértice, lo cual le confiere una abertura de ángulo de 20º. El resto de su estructura se compone de un entramado de hexágonos

similares a los existentes en una lámina de grafeno. Generalmente, este tipo de nanoestructuras se encuentran formando agregados estables con forma de dalhia los cuales presentan unos diámetros medios de 60-80 nm. Una particularidad de estas nanopartículas es la ausencia de catalizadores metálicos en su síntesis, lo cual hace que se obtengan nanocuernos de carbono libres de impurezas metálicas.

### II.1.5. Otros materiales

- Tubos de polipropileno (Deltalab, Barcelona, España).
- Cartuchos de extracción en fase sólida de 3 mL y tapones de PTFE suministrados por Análisis Vínicos (Tomelloso, España).
- Jeringas de plástico de 20 mL de Terumo (Heverlee, Bélgica).
- Barra magnética (4 mm x 25 mm y 42 N de fuerza) (Gottmadingen, Alemania).
- Bandeja de polipropileno 96 pocillos de 0.5 mL de Agilent (CA, USA).
- Tiras de 8 tubos de pared fina de 0.2 mL de Thermo Scientific (UK).

## II.2. MUESTRAS

Para evaluar la aplicabilidad de los distintos procedimientos de extracción desarrollados en esta Memoria de Tesis Doctoral en el ámbito medioambiental y clínico/farmacéutico, se seleccionaron muestras de agua y muestras biológicas, respectivamente.

Las muestras de agua una vez tomadas, se almacenaron en frascos de vidrio ámbar sin espacio de cabeza, en la oscuridad y a 4º C hasta su análisis. Se utilizaron muestras de agua de diferente procedencia: de grifo y mineral embotellada, de pozo, arroyo y río, y de piscina. Las muestras de agua de río pertenecen al río Guadalquivir. Asimismo, las muestras de agua de arroyo se recogieron de varios afluentes del río Guadalquivir a su paso por Córdoba y Jaén.

En cuanto a las muestras biológicas, se utilizaron biofluidos de distinta naturaleza: saliva, plasma y orina. Las muestras de saliva se obtuvieron de voluntarios que utilizaron una disolución de ácido cítrico para estimular su producción. Se recogieron en tubos de polipropileno y se congelaron a -18 ºC hasta su análisis. Con respecto a la muestras de plasma, éstas fueron proporcionadas por el Hospital Universitario de Oslo (Noruega), y se congelaron a -32 ºC hasta su análisis. Las muestras de orina se recogieron de voluntarios y se congelaron a -18 ºC. Antes de llevar a cabo el análisis de las mismas, se llevó a cabo un tratamiento con ácido y calor para poder cuantificar la fracción total de analito (libre + conjugado).

## II.3. INSTRUMENTOS

En el desarrollo experimental de la presente Tesis Doctoral se ha hecho uso de diferentes equipos instrumentales. Se pueden clasificar dividiéndolos en dos grandes grupos: los instrumentos empleados para la identificación y cuantificación de los analitos y los instrumentos usados para la caracterización de materiales sorbentes empleados.

Atendiendo a esta categorización, la instrumentación empleada en el análisis de los analitos fue:

- Cromatografía de gases acoplada a espectrometría de masas.
- Cromatografía de líquidos de alta presión con detector UV-visible.
- Cromatografía de líquidos de alta presión con detector UV-visible de diodos en fila.
- Cromatografía de líquidos de ultra-alta presión con detector UV-visible de diodos en fila.
- Cromatografía de líquidos de alta presión acoplado a espectrometría de masas.

Para la caracterización se ha utilizado:

- Espectroscopía infrarroja.

- Microscopía electrónica de barrido.

### II.3.1. Cromatografía de gases acoplada a espectrometría de masas (GC/MS)

Para la determinación de los compuestos orgánicos volátiles, se empleó un cromatógrafo de gases HP6890 de Agilent (Palo Alto, California, EEUU). Este equipo ofrece una alta versatilidad en su configuración ya que está diseñado para su acoplamiento a diferentes detectores como pueden ser: un detector de conductividad térmica, de ionización de llama o espectrómetro de masas. En la presente Tesis Doctoral se ha empleado el acoplamiento de cromatografía de gases a espectrometría de masas.

El inyector y el horno del cromatógrafo se programaron en términos de temperatura, presión y flujo, para realizar la separación bajo condiciones óptimas. Se seleccionó una columna capilar de sílice fundida HP-5ms (Supelco, Madrid, España) de 30 metros de longitud y 0.25 mm de diámetro interno, recubierta en su interior por una fase estacionaria de 0.25 µm de espesor de película y compuesta por un 5% de fenilpolisiloxano y un 95% de metilpolisiloxano. Como gas portador, se seleccionó helio de pureza 6.0 (Air Liquide, Sevilla, España) a un caudal de 1.4 mL/min que se fijó mediante un regulador digital de presión y flujo.

El cromatógrafo de gases se acopla mediante una línea de transferencia fijada a 250 ºC a un espectrómetro de masas HP5973 de Agilent (Palo Alto, California, EEUU), el cual se compone de una fuente de ionización de impacto electrónico, configurada para ionizar las moléculas procedentes del cromatógrafo de gases con una energía de ionización de 70 eV a una temperatura de 200 ºC. De esta manera, los espectros de masas obtenidos pueden compararse directamente con las diferentes bibliotecas estandarizadas de espectros existentes, lo cual permite la identificación de los analitos monitorizados. Además, este espectrómetro está equipado con un cuadrupolo como analizador de masas. Los cromatogramas se adquirieron en el modo SIM (Selected Ion Monitoring) estableciéndose

en cada caso los valores de m/z más convenientes para cada analito en sus correspondientes ventanas temporales.

Para llevar a cabo la desorción térmica de los compuestos volátiles, se hizo uso de un automuestreador de espacio de cabeza MPS2 (Gerstel, Mülhein y der Ruhr, Alemania). Este dispositivo está compuesto por un módulo de inyección en el que se encuentra una jeringa de gases de 2.5 mL que se mantuvo a una temperatura de 120 ºC; dos bandejas donde se colocan los viales con las muestras a analizar; un brazo robotizado para transportar tanto los viales como el módulo de inyección, y un horno que permite agitar y calentar las muestras para la generación del espacio de cabeza.

### II.3.2. Cromatografía de líquidos de alta presión acoplada a detección ultravioleta visible (HPLC-UV/Vis)

Se ha empleado un cromatógrafo de líquidos HP1100 de la casa Agilent compuesto por una bomba binaria de alta presión, un automuestreador, una columna LiChrosob® $C_{18}$ (4.6 mm x 150 mm) de Agilent y un detector ultravioleta/visible (HP1100 serie), para la determinación de paracetamol a 254 nm. Todo el sistema se controla por el software HP ChemStation, de la misma casa comercial.

### II.3.3. Cromatografía de líquidos de alta presión acoplada a detección ultravioleta visible de diodos en fila (HPLC-DAD)

Se ha utilizado un cromatógrafo de líquidos HP1200 de la casa comercial Agilent, compuesto por una bomba binaria de alta presión, un desgasificador, un automuestreador, un compartimento/horno para la columna y un detector ultravioleta/visible de diodos en fila (DAD). La separación cromatográfica de los anti-inflamatorios no esteroideos se llevó a cabo en una columna termostatizada de Syncronis $C_{18}$ con 3 µm de tamaño de partícula y de dimensiones 3 mm x 100 mm, de Thermo Fisher. Todo el sistema se controla por el software HP ChemStation, de Agilent.

### II.3.4. Cromatografía de líquidos de ultra-alta presión acoplada a detección de diodos en fila (UPLC-DAD)

Se ha empleado un cromatógrafo de líquidos Aquity$^{TM}$ de Waters, compuesto por una bomba de ultra alta presión, un desgasificador, un automuestreador, un compartimento/horno para la columna y un detector ultravioleta-visible de diodos en fila (DAD) para la determinación de analitos a varias longitudes de onda. Se ha utilizado una columna Aquity UPLC® BEH $C_{18}$ de 1.7 µm de tamaño de partícula, y de dimensiones 2.1 mm x 100 mm. El sistema se controla con el software Empore, de la misma casa comercial. Este equipo se ha empleado en dos problemas analíticos: (i) la determinación de benzofenona-3 y (ii) triazinas.

### II.3.5. Cromatografía de líquidos de alta presión acoplada a espectrometría de masas (HPLC-MS/MS)

Se ha utilizado un cromatógrafo de líquidos HP1260 de la casa comercial Agilent, compuesto por una bomba binaria de alta presión, un desgasificador, un automuestreador y un compartimento/horno para la columna. Se ha utilizado una columna Poroshell de Agilent 120 SB-$C_{18}$ (2.1 mm × 75 mm, 2.7 µm). El sistema cromatográfico está acoplado a espectrometría de masas de triple cuadrupolo con una fuente de ionización por electrospray (ESI). El software Agilent MassHunter WorkStation se usó para la toma de datos y el análisis cuantitativo. Esta instrumentación se ha empleado en dos problemas analíticos: (i) la determinación de triazinas en muestras acuosas y (ii) benzofenonas en muestras de orina.

### II.3.6. Espectroscopía infrarroja (IR)

La espectroscopía de infrarrojo se utilizó para la caracterización de la síntesis llevada a cabo en los discos de borosilicato con grupos octadecil. Las medidas de infrarrojo se realizaron en un espectrómetro FT-IR de Bruker modelo Tensor37 equipado con una unidad de reflectancia total atenuada (ATR) de diamante con una superficie circular de 3 mm de

diámetro y tres reflexiones internas. Los espectros fueron recogidos entre 4000 y 600 $cm^{-1}$ con resolución de 4 $cm^{-1}$ y 128 scans. Los datos se recogieron y trataron con el software OPUS (Bruker, Ettligen, Alemania).

### II.3.7. Microscopía electrónica de barrido (SEM)

La visualización de los nanocuernos de carbono inmovilizados sobre los discos de borosilicato, se llevó a cabo mediante microscopía electrónica de barrido (SEM) JEOL JSM 6300 (Isaza, Alcobendas, España) con una capacidad de aumento entre 70x y 300000x y una resolución de 3-4 nm (a 30 Kv).

El microscopio que se ha utilizado pertenece al Servicio Centralizado de Apoyo a la Investigación (SCAI) de la UCO.

### II.3.8. Otros instrumentos

- pH-metro (Crison, modelo micropH 2000), usado en la preparación de muestras, fases móviles y extractantes.
- Balanza analítica de precisión OHAUS Explorer (OHAUS, Nänikon, Suiza), empleada en la preparación de patrones y muestras.

## II.4. APARATOS

Durante el desarrollo del trabajo experimental realizado en esta Tesis Doctoral, se emplearon los siguientes aparatos:

- Agitadores:

  (i) Agitador magnético (Velp Científica, Milán, Italia) para llevar a cabo la agitación de los sistemas de extracción diseñados.

(ii) Agitador vórtex (Reax Top, Heidolph, Mérida, España) para la homogeneización de muestras y para agitar el sistema de extracción con membrana diseñado para muestras de disponibilidad limitada.

(iii) Plataforma agitadora (Vibramax 100, Heidolph, Schwabach, Alemania) utilizada para llevar a cabo la agitación en PALME.

- Baño de ultrasonidos 50 W, 60 Hz (J.P. Selecta, Barcelona, España) para desgasificar fases móviles y para llevar a cabo la dispersión y homogeneización de muestras y nanocuernos de carbono.
- Microondas de uso doméstico (AEG, Estocolmo, Suecia). Este microondas está equipado con un magnetrón de 2450 MHz y una potencia máxima de 800 W. Se utilizó para llevar a cabo la funcionalización de los nanocuernos de carbono.
- Ultracentrífuga controlada por microprocesador (Centronic BL-II, J.P. Selecta, Barcelona, España).
- Placa calefactora con agitación magnética Agimatic-N (J.P. Selecta, Barcelona, España).
- Sistema de extracción en fase sólida (Supelco, Bellefonte, PA) utilizado para la elución de los compuestos retenidos en los discos derivatizados.
- Equipo de agua Milli-Q (Millipore, Bedford, MA, EEUU).
- Soldador de hierro (Clas Ohlson AB, Insjon, Sweden), para sellar la membrana de polipropileno en PALME.
- Baño de agua con control de la temperatura (Selecta, Barcelona, España), usado para termostatizar el proceso de hidrólisis de los derivados glucurónicos en muestras de orina.

## II.5. DISEÑO DE UNIDADES DE MICROEXTRACCIÓN CON AGITACIÓN INTEGRADA

A continuación se exponen los diferentes sistemas de extracción/agitación diseñados fruto de la investigación recogida en la presente Memoria.

### II.5.1. Sistema de microextracción con frita

Las fritas de polietileno se han utilizado recientemente como herramientas en el desarrollo de procedimientos analíticos. Se han empleado como soporte para inmovilizar en sus poros materiales sorbentes, tales como materiales monolíticos [1] y polímeros de impresión molecular [2].

En el **Capítulo 1**, se presenta una alternativa para el uso de estas fritas, que ha dado lugar a una nueva técnica de extracción en la que se aprovechan y combinan las propiedades extractivas de este material (sin modificarlo químicamente) con la agitación integrada. Para ello, se perfora la frita con un pequeño alambre de hierro que permite la agitación magnética de la misma mientras tiene lugar la extracción, tal y como se muestra en la **Figura 6**.

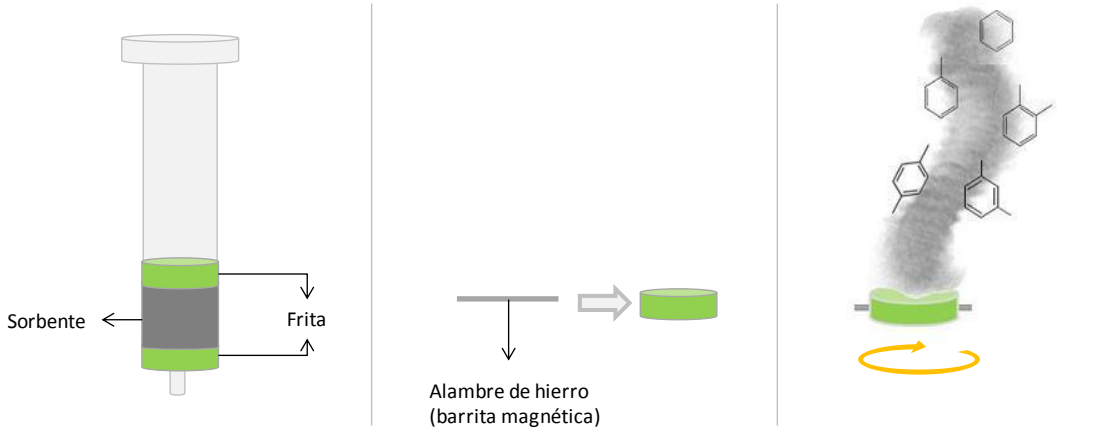


**Figura 6**. Microextracción con frita agitada.

Dado el carácter hidrofóbico de la frita de polietileno, este material está especialmente indicado para llevar a cabo el aislamiento y preconcentración de compuestos no polares. Concretamente, se llevó a cabo la determinación de compuestos orgánicos volátiles en muestras de agua. Una vez finalizada la etapa de extracción, la unidad se transfiere a un vial que se sella herméticamente y se coloca en el automuestreador de espacio de cabeza del GC/MS para que los analitos se desorban térmicamente.

### II.5.2. Sistema de microextracción basado en discos agitados

En los **Capítulos 2** y **6** se han utilizado discos de borosilicato como herramientas para el desarrollo de metodologías analíticas que permitan el aislamiento y la preconcentración de compuestos en matrices ambientales.

Los discos de borosilicato constituyen excelentes soportes para procesos analíticos dada su gran estabilidad mecánica y su estructura porosa. Además, son materiales muy versátiles ya que permiten la inmovilización de grupos funcionales de distinta naturaleza en su superficie. Antes de realizar la derivatización, es necesaria una etapa previa de activación mediante la cual se generen grupos hidroxilos superficiales, que serán posteriormente modificados covalentemente. Para llevar a cabo la activación, el disco se introduce en una mezcla de ácido sulfúrico/peróxido de hidrógeno a 100ºC durante un tiempo determinado.

En el **Capítulo 2**, los discos se modificaron con nanopartículas (NPs). Las excelentes propiedades sorbentes de las NPs han impulsado el desarrollo de metodologías basadas en estos nanomateriales, constituyendo excelentes soportes para procesos analíticos [3]. De entre todos los tipos de nanopartículas existentes, se optó por utilizar nanocuernos de carbono ya que, en comparación por ejemplo con nanotubos de carbono, son escasas las referencias en bibliografía que describan su potencial en el desarrollo de metodologías analíticas en el ámbito de la microextracción [4-5]. En la **Figura 7** se muestra el aspecto que presenta el disco de borosilicato perforado (A) antes y (B) después de la síntesis.

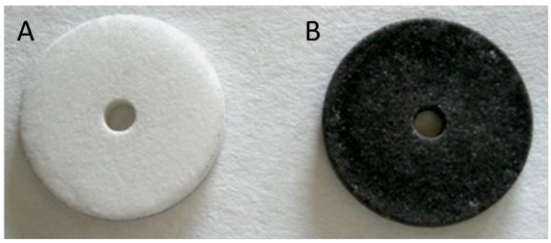


**Figura 7**. Fotografías de: A) disco sin modificar y (B) disco con nanocuernos oxidados de pared simple, inmovilizados sobre la superficie activa de sus poros.

Todas las etapas de la síntesis de este nuevo material sorbente se describen en detalle en el **Capítulo 2.** Los discos obtenidos se caracterizaron mediante SEM, observándose la formación de rugosidades sobre los poros, que se corresponden con dahlias de nanocuernos. Esta nueva metodología se ha aplicado a la determinación de benzofenona-3 en aguas de piscina.

El sistema de extracción, representado en la **Figura 8**, consta de cuatro elementos:

a) Disco modificado (20 mm de diámetro y tamaño de poro de 16-40 μm).
b) Tornillo.
c) Arandela.
d) Taladro.

En cuanto al proceso de ensamblaje, una vez sintetizado el disco, se perfora en el centro (con ayuda de un taladro manual), se introduce en el tornillo y se fija con una arandela. Este sistema se integra en un taladro, que hace posible su agitación.


**Figura 8**. Sistema de extracción propuesto de microextracción en fase sólida basado en nanocuernos de carbono oxidados inmovilizados sobre disco.

En el **Capítulo 6**, los discos (previamente activados) se modificaron con grupos octadecilo ($C_{18}$) para la determinación de triazinas en muestras de agua ambiental. Se trabajó en la modalidad de microextracción en fase líquida ya que las cadenas de $C_{18}$ se utilizaron para estabilizar mediante interacciones hidrofóbicas el disolvente orgánico no polar (extractante). Los discos obtenidos a través de este procedimiento de síntesis se caracterizaron por espectroscopía infrarroja y de forma visual mediante un sencillo ensayo colorimétrico con fenolftaleína. En cuanto al diseño del sistema de extracción, representado en la **Figura 9**, está constituido por tres elementos, siendo estos;

a) Disco derivatizado (20 mm de diámetro y tamaño de poro de 10-16 µm).
b) Sección superior de una jeringa de plástico de 20 mL (20 mm de diámetro y 15 mm de altura).
c) Barra magnética de dimensiones 4 mm x 25 mm.

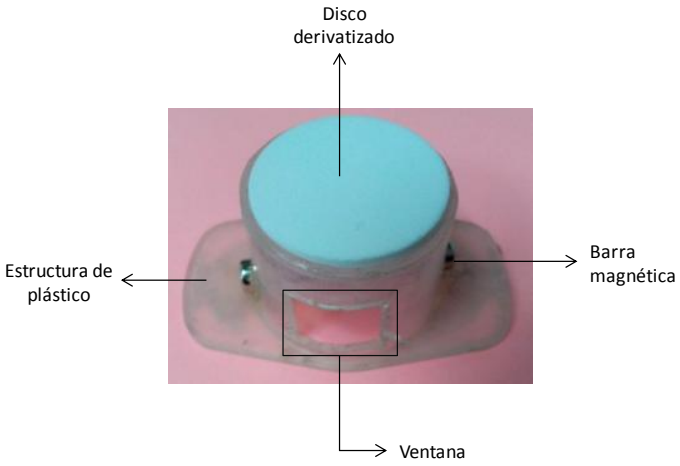


**Figura 9.** Elementos que integran el sistema agitado con disco de $C_{18}$.

El proceso de ensamblaje se describe a continuación. En primer lugar, se corta una sección de la jeringa y se realizan cuatro perforaciones (ventanas) enfrentadas dos a dos. A continuación se introduce por dos de ellas la barra magnética recubierta previamente de cloruro de polivinil que previene su oxidación. Las otras dos ventanas creadas favorecen el flujo de materia. Se coloca el disco sobre la unidad y es importante que ambas piezas encajen perfectamente. Antes de realizar la extracción, se impregna el disco derivatizado con el disolvente orgánico para formar la membrana líquida soportada, que como se ha descrito anteriormente, es la responsable de la extracción de los compuestos.

### II.5.3. Sistema de microextracción basado en fases extractivas sintéticas

Las fases extractivas sintéticas, más conocidas como *fabric phase*, han sido desarrolladas por los Prof. Kenneth G. Furton y Prof. Abuzar Kabir [6], del Instituto Internacional de Investigación Forense del Departamento de Química y Bioquímica de la Universidad Internacional de Florida (Florida, USA). Estos materiales se crean utilizando un sustrato flexible (plataforma) sobre el que se sintetiza un recubrimiento híbrido orgánico-inorgánico (sorbente) mediante la tecnología sol-gel [7]. Se trata de un material novedoso que

presenta un gran potencial en la preparación de muestras y en el desarrollo de procedimientos analíticos, supliendo algunas de las carencias de la microextracción en fase sólida convencional como la inestabilidad de algunos recubrimientos, la limitación de formatos disponibles o la irreproducibilidad en la síntesis de diferentes lotes. Las *fabric phase* se caracterizan por presentar:

(i) Elevada porosidad, lo que facilita un mayor flujo de muestra a su través, mejorando la partición de analitos.
(ii) Elevada capacidad de extracción.
(iii) Alta estabilidad debido a que el sorbente se encuentra fuertemente unido a la plataforma mediante enlaces covalentes. Por ello, pueden ser expuestas a cualquier disolvente o mezcla de varios disolventes sin dañarse ni sufrir modificaciones en su estructura. De igual forma, son estables en un intervalo amplio de pH.
(iv) Distribución homogénea del sorbente.
(v) Alta versatilidad, ya que abarcan un gran intervalo de polaridad. Además, existe gran variedad en cuanto a las configuraciones geométricas se refiere.

Hasta la fecha, únicamente se ha aplicado a la determinación de estrógenos en muestras de orina [8] y anfenicoles en leche [9], ambas metodologías desarrolladas por el grupo del Prof. Furton. La configuración del dispositivo original, mostrado en la **Figura 10**, pone de manifiesto la utilización de un elemento externo para llevar a cabo la agitación.


**Figura 10**. Fotografía del sistema de extracción original. Reproducido de la Ref. 8.

De esta manera, en los **Capítulos 4** y **5** se combina la excelente capacidad extractiva de las *fabric phase* con el beneficioso efecto de la agitación. Para ello, se utilizó la configuración del sistema de extracción con membrana agitada [10], reemplazando la membrana polimérica por la *fabric phase*. En el **Capítulo 4**, el sistema de extracción se ha caracterizado para la determinación de triazinas en muestras de agua ambientales, y en el **Capítulo 5** se han determinado benzofenonas en muestras de orina.

Los cuatro elementos básicos que integran la unidad de extracción se representan en la **Figura 11**; siendo estos:

a) Sección superior de un cartucho comercial de SPE de 3 mL (1 cm de diámetro interno y 0.6 cm de altura), que se corresponde con la parte interna.
b) Una sección de una punta de pipeta de 5 mL (1.2 cm de diámetro interno y 0.5 cm de altura), que es la parte externa.
c) *Fabric phase*.
d) Barra agitadora (1.4 cm de longitud).

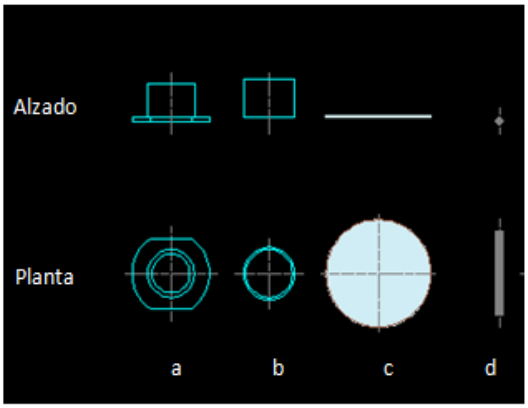


**Figura 11**. Elementos básicos que componen la unidad de extracción con *fabric phase* agitada.

En cuanto al proceso de ensamblaje, la fase extractiva se coloca en la parte superior del cilindro interno, sellándose finalmente la unidad por desplazamiento de la parte externa sobre la interna. Las dimensiones de ambos cilindros son críticas para asegurar que la *fabric phase* quede bien fijada, evitando así cualquier desplazamiento de la misma durante las extracciones. Por último, la unidad de extracción se perforó con un alambre de hierro para permitir la agitación magnética del dispositivo. La configuración final se muestra en la **Figura 12**.

**Figura 12**. Fotografía de la unidad de extracción con *fabric phase* agitada.

### II.5.4. Sistemas de microextracción en tres fases

En el sistema de tres fases, los analitos se extraen desde una fase acuosa (fase donadora) a través del disolvente orgánico inmovilizado en los poros de la membrana (fase orgánica) hasta otra fase acuosa (fase aceptora). La fase orgánica en este caso, actúa como una barrera entre las disoluciones acuosas donadora y aceptora, impidiendo la mezcla de ambas fases.

Esta técnica se aplica a analitos que presentan grupos ionizables en su estructura y cuya carga depende del pH de trabajo. De hecho, el gradiente de pH que se establece a ambos lados de la capa de disolvente orgánico es crucial ya que es la fuerza impulsora de la extracción. En este sentido, el pH de la muestra se ajusta con objeto de transformar los

analitos en su forma neutra, induciendo así su paso a la fase orgánica. La fase acuosa aceptora se ajusta a un pH en el que los analitos estén en su forma iónica, consiguiendo así que pasen desde el disolvente orgánico. Dependiendo de la naturaleza de los compuestos, se pueden establecer diferentes gradientes de pH. Para la extracción de analitos básicos, el pH de la muestra se debe ajustar en la región alcalina mientras que el de la fase aceptora debe ser ácido. En el caso de analitos ácidos, se acidifican las muestras y se seleccionan fases aceptoras alcalinas.

Este tipo de preparación de muestra, proporciona extractos limpios compatibles con cromatografía de líquidos, ya que:

(i) El carácter poroso de las membranas puede emplearse como un paso limitante para aquellos compuestos que tienen un tamaño superior al del poro (evitando el paso de biomoléculas, en el caso de muestras biológicas).

(ii) Este procedimiento está limitado a la extracción de compuestos ionizables de naturaleza hidrofóbica, aunque podría emplearse con compuestos polares si se utilizan moléculas transportadoras (carriers) en el sistema.

Se han desarrollado dos sistemas de microextracción líquido-líquido-líquido (tres fases) para la determinación de fármacos en biofluidos. El primer sistema de extracción propuesto, descrito en el **Capítulo 7**, está inspirado en la técnica de microextracción con membrana líquida agitada [11]. Este dispositivo se caracteriza por la separación y preconcentración de los analitos de un modo simple y eficiente, mejorando la sensibilidad de las determinaciones y la selectividad de las mismas.

Se ha adaptado al análisis de muestras de disponibilidad limitada, llevando a cabo la determinación de paracetamol en saliva. Para esta aplicación, se modificó el diseño de la unidad de extracción original con el objetivo de reducir el volumen de muestra a unos pocos mililitros. La unidad de extracción, está constituida por cuatro elementos básicos (**Figura 13**):

a) Un tapón comercial de PTFE para los cartuchos de SPE de 3 mL.
b) Sección superior de un cartucho comercial de SPE de 3 mL (1 cm de diámetro interno y 0.6 cm de altura).
c) Membrana de PTFE (100 µm de espesor).
d) Una sección de una punta de pipeta (1.2 cm de diámetro interno y 4 cm de altura).

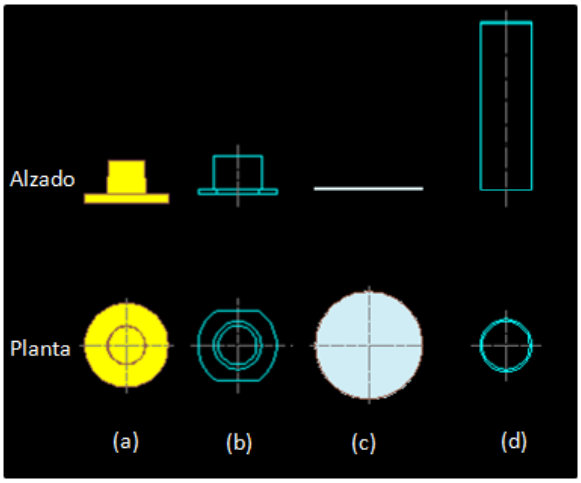


**Figura 13**. Elementos básicos que componen la unidad de microextracción líquida con membrana agitada.

El proceso de ensamblaje se esquematiza en la **Figura 14** y es el siguiente. En primer lugar, el elemento interno (b) se coloca sobre el tapón comercial de PTFE (a) y se sella por presión. El ensamblaje de los elementos a y b es crucial, ya que definen la cámara interna (50 µL) donde se sitúa la fase aceptora. A continuación, se pipetea la fase aceptora y se coloca la membrana de teflón sobre la unidad, fijándola por desplazamiento del elemento externo (d). La membrana se impregna con un pequeño volumen de disolvente orgánico para así formar la membrana líquida soportada, que separa a la muestra y a la fase aceptora.

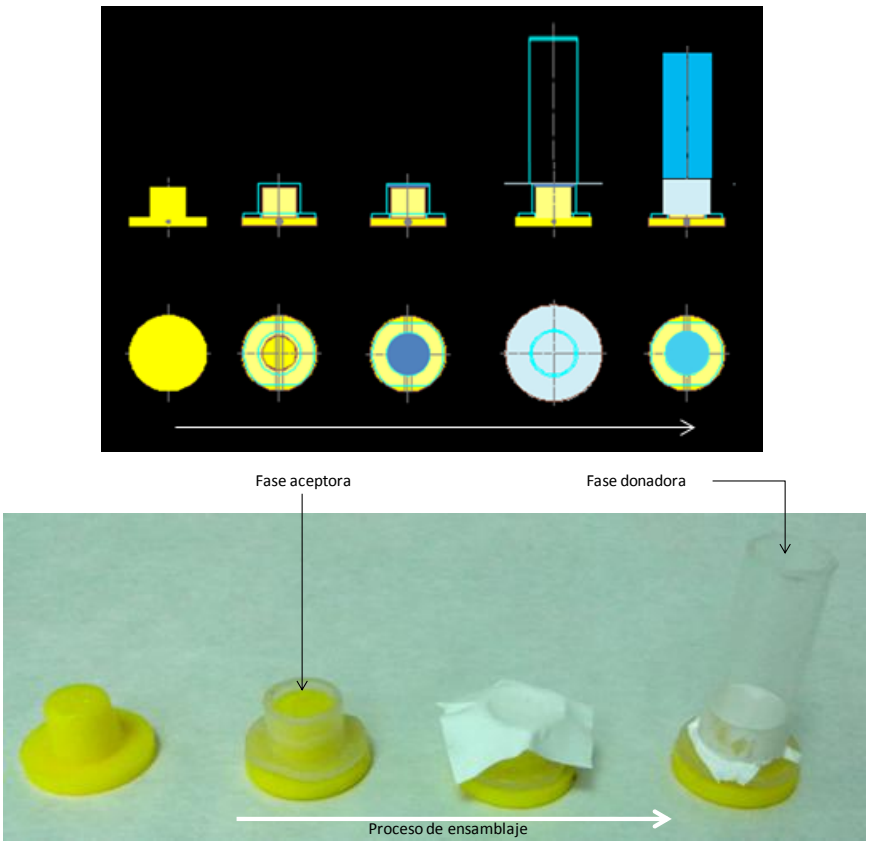


**Figura 14.** Esquema y fotografía del proceso de ensamblaje de una unidad de microextracción líquida con membrana agitada.

En la **Figura 15** se observa que en el dispositivo diseñado se incrementa la altura del elemento externo con respecto al original, generando una cámara que alberga a la muestra. De esta manera, la muestra se encuentra integrada en la unidad de extracción. En cuanto al sistema de agitación, en este caso no se utilizó alambre de hierro porque el sistema era inestable, así que se optó por llevar a cabo la agitación en un vórtex.

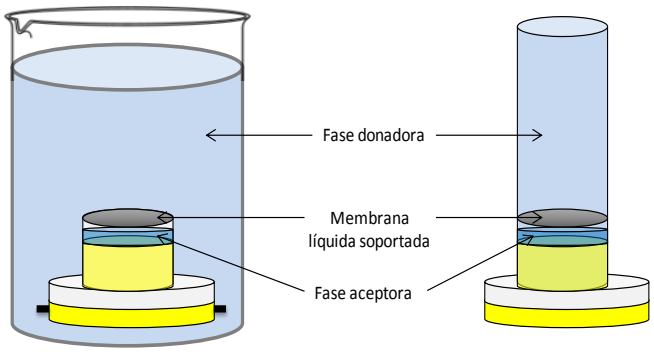


**Figura 15.** Comparación de sistemas de microextracción en tres fases con membrana agitada. A la izquierda, dispositivo inicial. A la derecha, la modificación propuesta para el tratamiento de muestras de volumen limitado.

El segundo sistema de extracción utilizado, descrito en el **Capítulo 8**, ha sido desarrollado recientemente por el grupo de investigación del Prof. Pedersen-Bjergaard [12]. Se trata de un sistema de extracción con membrana líquida artificial en paralelo, más conocido como PALME, por sus siglas en inglés. Este dispositivo se caracterizó para la determinación de anti-inflamatorios (compuestos de naturaleza ácida) en muestras de plasma. El sistema de extracción, consta de cuatro elementos comerciales (**Figura 16**):

a) Una bandeja con 96 pocillos de 0.5 mL.
b) Tiras de 8 tubos de pared fina de 0.2 mL.
c) Membrana de PP (100 µm de espesor).
d) Una tapadera.

El proceso de ensamblaje es el siguiente. En primer lugar, se crea la cámara que albergará a la fase aceptora. Para ello, la membrana (c) se fija a la serie de 8 tubos recortados (b) mediante un soldador a alta temperatura. A continuación, la muestra se pipetea sobre los pocillos de la bandeja (a) y la cámara para la fase aceptora con la membrana sellada se colocan sobre ellos. Seguidamente, se impregnan las membranas con 2 µL de disolvente orgánico y se añade la fase aceptora. Por último, es importante colocar una tapadera (d)

que encaje sobre el sistema para cerrarlo y evitar pérdidas por evaporación de la fase aceptora. El sistema se agita en una plataforma vibradora.

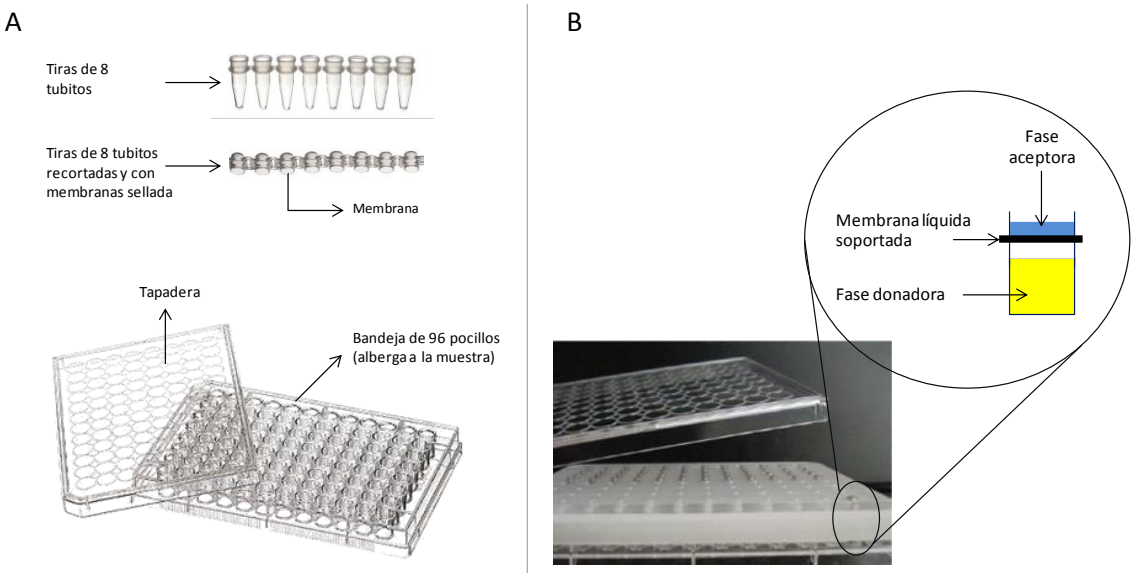


**Figura 16.** Fotografías de: (A) los elementos que integran PALME y (B) de la configuración final del sistema, con detalle de un pocillo.

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BLOQUE III

# MICROEXTRACCIÓN EN FASE SÓLIDA CON UNIDADES AGITADAS

**Capítulo 1**

Stir frit microextraction: An approach for the determination of volatile compounds in water by headspace –gas chromatography/mass spectrometry.

**Capítulo 2**

Micro-solid phase extraction based on oxidized single walled carbon nanohorns immobilized on a stir borosilicate disk: Application to the preconcentration of the endocrine disruptor benzophenone-3.

**Capítulo 3**

Solid phase microextraction under the thin film format.

**Capítulo 4**

Stir fabric phase sorptive extraction for the determination of triazine herbicides in environmental waters by liquid chromatography.

**Capítulo 5**

Stir fabric phase sorptive extraction for the determination of benzophenone-type filters in urine.

La extracción en fase sólida (*solid phase extraction*, SPE) surgió para suplir las limitaciones que la extracción líquido-líquido convencional (*liquid-liquid extraction*, LLE) presenta. La tendencia de la SPE hacia metodologías más selectivas, simples y miniaturizadas ha dado lugar a la microextracción en fase sólida (*solid phase microextraction*, SPME), propuesta por Pawliszyn en 1990. En 1999, el grupo del profesor Sandra desarrolló la microextracción en barrita sorbente agitada (*stir bar sorptive extraction*, SBSE). Esta modalidad es una alternativa a la técnica de SPME convencional con la que comparte los mismos fundamentos. Sin embargo, la SBSE presenta una serie de ventajas frente a la SPME: (i) la cantidad de fase extractiva utilizada es considerablemente mayor (del orden de 50 a 250 veces), lo que implica una mejora de la sensibilidad; y (ii) en el diseño del sistema, la extracción y la agitación se encuentran integradas, lo que mejora la eficiencia. La evolución de la SBSE va encaminada a la mejora del procedimiento de tratamiento de muestra, estando materializada en dos líneas de investigación complementarias:

(i) El desarrollo de nuevos materiales para solventar la principal limitación de la SBSE: la falta de una gran gama de fases extractivas. Durante años, la única barrita sorbente comercializada ha sido de polidimetilsiloxano (PDMS) (Twister®), lo cual limitaba el uso de esta técnica a la extracción de compuestos no polares. Con el objetivo de ampliar su campo de aplicación y hacer posible la extracción de compuestos polares, actualmente se comercializan barritas de silicona modificadas con polietilenglicol (EG Silicone Twister®) y también se están haciendo pruebas con poliacrilato combinado con polietilenglicol (Acrylate Twister®). Por otro lado, se trabaja también en nuevos recubrimientos con polímeros de impresión molecular (MIPs), materiales de acceso restringido (RAMs), espuma de poliuretano (PU) y monolíticos, entre otros.

(ii) El desarrollo de nuevas configuraciones que integren la extracción y la agitación en el mismo dispositivo. El objetivo es la eliminación de los problemas asociados a la inestabilidad mecánica del recubrimiento de la SBSE, los cuales vienen

principalmente originados por el rozamiento de la barrita con el fondo del vial donde se realiza la extracción.

En este Bloque de la Memoria se presentan las metodologías de extracción en fase sólida desarrolladas, que combinan la extracción y la agitación en el mismo dispositivo y que están basadas en el uso de sorbentes no convencionales. Se detallan también los diseños propuestos para explotar y potenciar las propiedades extractivas de dichos materiales.

La evaluación del uso de fritas de polietileno como medio sorbente se describe en el **Capítulo 1**. La frita se perfora con un pequeño alambre de hierro que permite la agitación magnética de la misma mientras tiene lugar la extracción de benceno, tolueno, estireno, isómeros del xileno y etilbenceno en muestras de agua. Se lleva a cabo la desorción térmica de los compuestos aromáticos retenidos en la frita agitada y su posterior análisis mediante cromatografía de gases acoplada a espectrometría de masas.

En el **Capítulo 2** se ha evaluado la capacidad sorbente de los nanocuernos de carbono inmovilizados en los poros de un disco de borosilicato. Las características sorbentes que presentan estas nanopartículas hacen posible su uso como herramientas analíticas en el ámbito de las técnicas de extracción miniaturizadas. Además, se describe la versatilidad que presentan los discos de borosilicato como soporte para ser utilizados en procedimientos analíticos. En cuanto a la inmovilización, ésta consta de tres etapas: (i) activación del disco de borosilicato para la creación de grupos silanoles, (ii) funcionalización de los nanocuernos de carbono de pared simple, llevada a cabo empleando energía de microondas que introduce en su estructura grupos funcionales oxigenados y finalmente (iii) inmovilización de los nanocuernos funcionalizados sobre el disco activado. El disco resultante se integra en un taladro que hace posible su agitación durante la extracción. Cabe destacar que en esta configuración se plantea el potencial de utilizar esta herramienta para realizar extracciones *in situ*. El problema analítico modelo seleccionado fue la determinación de benzofenona-3 en aguas de piscina. Como técnica de separación se

utiliza la cromatografía líquida de ultra alta presión acoplada a detector de diodos en fila ultravioleta/visible.

Por otro lado, en este Bloque se presenta una revisión de la microextracción en capa fina (*thin film microextraction*, TFME) (**Capítulo 3**). Esta técnica de microextracción en fase sólida se desarrolló para lograr una mayor eficiencia y sensibilidad en las extracciones. A diferencia de la SPME en fibras, existe una mayor superficie de contacto entre las membranas empleadas en TFME y la muestra, lo que da lugar a extracciones más rápidas y efectivas. Este capítulo sirve de guía para contextualizar las distintas configuraciones existentes en este ámbito. Se describen también los materiales emergentes que han sido recientemente desarrollados para llevar a cabo la TFME.

En este sentido, en el **Capítulo 4** se propone el uso de *fabric phase* en un sistema rotatorio para la determinación de triazinas de muestras de agua ambientales. Para ello, se utilizó la configuración del sistema de extracción con membrana agitada, reemplazando la membrana polimérica por la *fabric phase*. Como técnica de separación y detección se utiliza la cromatografía líquida de ultra alta presión acoplada a detector de diodos en fila ultravioleta/visible. Se compara también la técnica de extracción propuesta con otras metodologías existentes para la determinación de triazinas en muestras de agua. Finalmente, se realiza una validación mediante cromatografía de líquidos y espectrometría de masas ya que es la técnica de referencia en este ámbito.

En el **Capítulo 5** se evalúa el uso de *fabric phase* como medio sorbente en la extracción en fase sólida de cinco benzofenonas en muestras biológicas (orina). Es necesario llevar a cabo la hidrólisis de los metabolitos excretados para poder cuantificar la cantidad total excretada del analito exógeno de partida. Asimismo, se realiza una dilución para evitar los potenciales interferentes de la matriz. En este trabajo el estudio de las variables experimentales se llevó a cabo mediante cromatografía líquida de ultra alta presión acoplada a detector de diodos en fila ultravioleta/visible y en la validación de la metodología propuesta se utiliza cromatografía líquida acoplada a espectrometría de masas.

**CAPÍTULO 1**

# Stir frit microextraction: An approach for the determination of volatile compounds in water by headspace –gas chromatography/mass spectrometry

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# Stir frit microextraction: An approach for the determination of volatile compounds in water by headspace-gas chromatography/mass spectrometry

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In this article, a novel extraction approach, called stir frit microextraction (SFME), is presented. The new approach combines the extractive capability of a commercial polyethylene frit (20 μm of pore size) with the stirring in the same device. The proposed extraction procedure allows the determination of benzene, toluene, ethylbenzene, xylene isomers and styrene (BTEX-S) in water samples. The analytes are extracted on the frit, previously conditioned with methanol, under continuous magnetic stirring. Once the extraction is performed, the frit is transferred to a headspace vial where the volatile compounds are desorbed from the frit (90 ºC, 30 min) in a headspace module and analyzed by gas chromatography/mass spectrometry. Headspace conditions (time and temperature) as well as extraction conditions (ionic strength, type of stirring, extraction time, stirring rate and sample volume) have been systematically evaluated. The method was characterized on the basis of its linearity, sensitivity and precision. Limits of detection were in the range from 18 ng/L (*o*-xylene) to 65 ng/L (benzene). The repeatability of the proposed method, expressed as relative standard deviation (RSD) varied between 3.8% (toluene) and 8.2% (*m*- and *p*-xylene). The recovery study carried out in different water samples provided an average recovery of 94%, which demonstrated the applicability of the stir frit microextraction for the analytical problem selected in this article.

***Keywords:*** *Stir frit microextraction; BTEX-S; Water samples; Headspace; Gas chromatography; Mass spectrometry.*

## 1. INTRODUCTION

Stir bar sorptive extraction (SBSE), which was introduced in 1999 [1], shares almost the same principles of solid phase microextraction (SPME) [2,3] since both are based on the partitioning of the target analytes between the sample and a sorptive material immobilized on an inert support. In SPME the stirring and extraction elements are independent, which may be problematic when excessively non-polar compounds are considered due to their potential retention on the polymeric bar used for sample stirring [1]. This limitation is overcome in SBSE since it integrates both elements in the same device. Moreover, the extracting phase volume is 50–250 higher in SBSE [4] enhancing therefore the extraction recoveries.

Classic SBSE is mainly focused on the extraction of non-polar compounds from aqueous samples which is a direct consequence of the first stir bar commercialized under the name of Twister®. This stir bar consists of a glass jacketed metal bar coated with a polydimethylsiloxane (PDMS) coating which is characterized by its non-polar nature. In fact, the octanol/water constant ($K_{o/w}$) is usually employed to define the partition of a given analyte between a water solution and a PDMS coating ($K_{o/w} \approx K_{PDMS/w}$) [5].

Dedicated desorption units that can be integrated in the chromatograph are also commercially available. Although new commercial coatings like polyacrylates are available [6], their limited number is a clear restriction in the extension of the SBSE principles to more polar analytes. In this sense, PDMS modified coatings [7, 8] based on the addition of special modifiers to PDMS, have been proposed for this purpose with excellent results. On the other hand, monolithic materials can be also considered as promising coatings since a wide range of polarity can be achieved just selecting the appropriate monomers for their synthesis [9–11]. These lab-made coatings are characterized by a porous structure, allowing the increasing of the extracting phase volume with a negligible negative effect both on the extraction and elution kinetics. Finally, other coatings including molecularly imprinted

polymers [12–14], restricted access materials [15] or polyurethane foams [16] have also been proposed in this framework.

The development of new extraction procedures that integrate the extraction and stirring element in the same device has become a promising research field in order to improve some practical aspects of classic SBSE or to allow the use of the novel coatings.

In this context, Alcudia *et al.* proposed for the first time in 2009 the so-called stir membrane extraction (SME), which uses a polymeric membrane as extracting phase in a novel stirring unit [17]. The general approach has been also adapted to the liquid microextraction format bringing on the development of the stir membrane liquid–liquid microextraction (SM-LLME) under the two phase [18] and three phase modes [19].

Rotating-disk sorptive extraction (RDSE) [20] proposed by Richter *et al.* in 2009 allows the use of higher stirring velocities than classic SBSE since the PDMS coating is disposed in the form of a thin film and protected from the vessel bottom by a polytetrafluoroethylene (PTFE) disk. On the other hand, the stir rod sorptive extraction (SRSE) [21] and stir cake sorptive extraction (SCSE) [22] emerged to face up the cracking tendency of monolithic materials by avoiding their direct contact with the vessel walls or protecting them with a plastic body, respectively.

Polyethylene (PE) frits have been recently employed as inert supports for the immobilization of sorptive materials in solid phase extraction (SPE). In this sense, monolithic material [23] and molecularly imprinted polymers [24] have been successfully grafted in the frit pores.

In this article, a novel microextraction which combines the inherent extractive capability of raw PE frits with the advantages of stirring is presented. The novel approach has been practically evaluated using BTEX-S (benzene, toluene, ethylbenzene, xylenes and styrene) as analyte models, using headspace coupled with gas chromatography/mass spectrometry as

instrumental technique. The variables involved in the extraction process, as well as the instrumental conditions, were identified and conveniently optimized.

## 2. EXPERIMENTAL

### 2.1. Reagents, materials and samples

All the reagents were of analytical grade or better. The analytes, BTEX-S: benzene, toluene, ethylbenzene, *m*-, *p*-, *o*-xylenes and styrene were supplied by Sigma–Aldrich (Madrid, Spain). Stock standard solutions of the analytes were prepared in methanol (Panreac, Barcelona, Spain) at a concentration of 1 g/L and stored in the dark at 4 ºC. Working solutions were prepared by a rigorous dilution of stock solutions with methanol or Milli-Q ultrapure water (Millipore Corp., Madrid, Spain) as required.

Sodium chloride and *o*-xylene-$d_{10}$, both from Sigma–Aldrich, were used to adjust the ionic strength and as internal standard, respectively.

PE frits (20 µm of pore size) from 3 mL SPE cartridges, provided from Análisis Vínicos (Tomelloso, Spain), were employed for the construction of the extraction units. Each frit was pierced with an iron wire (1 cm) to allow the magnetic stirring of the device.

Tap and well water samples were collected in amber-glass bottles without headspace. Bottled water samples were purchased in a local market. The samples were stored in the dark at 4 ºC until their analysis.

### 2.2. Apparatus

Headspace analyses were performed on a MPS2 32-space headspace autosampler (Gerstel, Mülhein an der Ruhr, Germany) including a robotic arm and an oven. An automated injector fitted with a 2.5 mL gastight HS-syringe (heated at 120 ºC) was used for the introduction of

0.5 mL of the homogenized headspace from the vial into the HP6890 gas chromatograph (Palo Alto, CA, USA) equipped with a HP5973 mass spectrometer based on a quadrupole analyser and an electron multiplier detector.

The analytes were separated on a HP5MS fused silica capillary column (30 m × 0.25 mm i.d.) coated with 5% phenylmethyl-polysiloxane (film thickness 0.25 μm) (Supelco, Madrid, Spain). The temperature program of the chromatographic oven began at 40 ºC for 3 min, raised up to 80 ºC at 5 ºC/min, ramped at 50 ºC/min up to 200 ºC and kept finally at 200 ºC for 3 min. A column split ratio of 1:10 was selected for injection. Helium (6.0 grade purity, Air Liquide, Seville, Spain), at a flow rate of 1 mL/min, regulated by a digital pressure and flow controller, was used as carried gas. Electron impact ionization (70 eV) was used for analyte fragmentation. The MS source and quadrupole temperatures were maintained at 230 and 150 ºC, respectively. The chromatograms were acquired and processed by using G1701BA Standalone Data Analysis software (Agilent Technologies) on a Pentium 4 computer, which also controlled the whole system.

The mass spectrometer detector operated in selected ion monitoring mode recording the following m/z fragment-ions in a single window: 77, 78, 91, 92, 104, 106 and 116. The analytes were identified according to their retention times since the whole procedure (involving an extraction, headspace analysis and chromatographic separation) presents a high selectivity. The quantification was performed considering the characteristic ions of the analytes (77 and 78 for benzene, 91 and 92 for toluene, 91 and 106 for ethylbenzene, 91 for *p*- and *m*-xylene) although for the determination of *o*-xylene and styrene, which overlapped, specific m/z fragments (91 and 106 for *o*-xylene and 104 for styrene) were extracted and the corresponding ions chromatograms were integrated for quantification purposes. Moreover, *o*-xylene-$d_{10}$ (m/z 116) was used as internal standard in order to improve the precision of the whole procedure.

### 2.3. Analytical procedure

The proposed extraction procedure, which is based on the principles of solid phase extraction, is as follows: 100 mL of the aqueous standard solution or sample containing the target analytes, the internal standard (at 1 µg/L) and 100 g/L of sodium chloride are added to a 100 mL volumetric flask and placed in a magnetic stirrer (Velp Cientifica, Milan, Italy). Then, a PE frit, previously wet with methanol (ca. 55 µL), is introduced into the volumetric flask.

In this case, methanol acts as conditioning solvent making possible the close contact between the PE frit and the analytes. The whole system is stirred at 900 rpm for 30 min to perform the extraction of the analytes on the frit. Once finished, the frit is placed into a 10 mL headspace vial, which is hermetically sealed with a silicone septum and placed into the autosampler. The robotic arm takes each vial from the tray and places them into the oven where the frit is heated at 90 ºC for 30 min under mechanical stirring at 750 rpm to ensure the quantitative transference of the analytes to the headspace of the vial. After that, 0.5 mL of the headspace is introduced with a syringe (heated at 120 ºC) into the gas chromatograph/mass spectrometer, where the separation and identification/quantification of the analytes take place.

## 3. RESULTS AND DISCUSSION

All the variables involved in the extraction procedure were identified and optimized following a one variable at time approach, in order to isolate the effect of each variable on the new proposed method. The optimization process was performed by using standard solutions containing all the analytes at a concentration of 50 µg/L.

The variables involved, including instrumental and experimental ones, as well as the interval studied and the optimum values, are presented in **Table 1**. The initial values for all the

variables at the beginning of the optimization process are also indicated. When a variable is optimized, its optimum value is fixed for further studies.

The extraction device was a PE frit (commonly used as filter in SPE) pierced with an iron wire allowing the extraction of the analytes and the stirring in the same device. Therefore, no external stir bar was employed.

**Table 1**. List of the variables involved in the proposed extraction technique.

| Variable | Initial value | Studied values | Optimal value |
|---|---|---|---|
| **INSTRUMENTAL** | | | |
| Desorption time (min) | | 5, 15, 30, 45, 120 | 30 |
| Desorption temperature (ºC) | 100 | 70, 90, 100, 110 | 90 |
| Headspace volume injected (mL) | 1 | 0.5, 1, 2.5 | 0.5 |
| **EXPERIMENTAL** | | | |
| Ionic strength (NaCl g/L ) | 0 | 0, 50, 100, 200 | 100 |
| Extraction time (min) | 15 | 5, 15, 30, 45, 60 | 30 |
| Stirring mode | Mechanical | Mechanical, ultrasound, magnetic | Magnetic |
| Stirring rate (rpm) | 750 | 0, 100, 300, 500, 750, 900 | 900 |
| Sample volume (mL) | 10 | 10, 20, 50, 100, 250 | 100 |

The conditioning of the frit seems to play an important role in the extraction procedure. Different organic solvents (acetone, acetonitrile, methanol and hexane) were evaluated in order to cover a wide range of polarity. For this purpose, before the extraction the PE frit was immersed into the corresponding solvent, being dipped with ca. 55 µL. Higher analytical signals (between 2 and 4 times depending on the analyte) were obtained when

acetone, acetonitrile or methanol were employed in comparison with the extraction performed without dipping the frit. In fact, negligible differences were observed for these solvents. However, hexane was less suitable to this application since it provides many interferent peaks in the final chromatogram.

### 3.1. Evaluation of the headspace conditions

The transference of the analytes from the sorbent to the headspace of the vial, is a critical step in the methodology and it depends on two main variables, namely: desorption time and temperature. The effect of the desorption time, was studied in the range from 5 to 120 min, showing a similar behavior for all the analytes. In this sense, the analytical signal increased with the temperature up to 30 min, a slightly increase was observed for longer times. As a compromise between the analysis time and the sensitivity, 30 min was selected as the optimum value for further studies. On the other hand, desorption temperature was evaluated in the range from 70 to 110 ºC. In this case, the peak areas of the analytes increase with the temperature, this tendency being more marked for the more volatile compounds. However, at higher temperatures an unexpected diminution is observed, which can be ascribed to the negative effect of the temperature on the PE frit porosity. According to these results, 90 ºC was selected as the optimum desorption temperature for subsequent analysis to ensure that all the compounds were efficiently desorbed from the frit.

Finally, the effect of the headspace volume injected in the chromatograph was evaluated in the range from 0.5 to 2.5 mL. Although the peak areas for all analytes increased with the volume injected, the chromatographic resolution would be affected when larger volumes of sample are processed. Therefore, the optimum value was fixed at 0.5 mL since it provides an acceptable sensitivity level. Moreover it allows the extraction of larger volume of samples, which is essential in order to evaluate the potential enrichment capacity of the new extraction mode.

## 3.2. Evaluation of the extraction conditions

### *3.2.1. Ionic strength*

Ionic strength enhanced the extraction capability of the device by salting-out effect. This variable was evaluated using sodium chloride as model electrolyte varying its concentration in the sample in the range from 0 to 200 g/L. In general, the peak areas increase with increasing salt concentration (data not shown) in the aqueous sample up to 100 g/L, remaining almost constant for higher concentrations. Consequently, the results indicated that the addition of salt enhances the extraction and a concentration of 100 g/L was selected for further studies.

### *3.2.2. Effect of the type of stirring and extraction time*

The extraction time was evaluated in the interval from 5 to 60 min using three different types of frit stirring, namely: (1) mechanical, (2) ultrasound and (3) magnetic. Mechanical stirring was performed at 750 rpm in the headspace module of the equipment working in the off-line mode and maintained at room temperature. Ultrasound stirring was performed in an ultrasonic bath (50 W, 60 Hz) while magnetic stirring was performed with the proposed stir frit unit. In the latter approach, the frit is pierced by an iron wire and an external magnetic stirrer is employed. For simplicity, the results obtained for three representative analytes are shown in **Figure 1**. Benzene, toluene and styrene are represented in order to cover a wide range of volatilities. When mechanical stirring was employed, analytical signals increased with the extraction time between 5 and 30 min remaining almost constant for longer times. A similar behavior was observed for magnetic stirring. On the other side, when ultrasound was employed to assist the extraction, the peak areas of the analytes continuously increased with the extraction time in the range from 5 to 60 min. Magnetic stirring provides higher analytical signals for all the analytes which indicates the good performance of the integration of stirring and extraction in the same device. In fact, it provides an efficient stirring avoiding a potential temperature increase

that is problematic in extraction techniques. According to the results, 30 min of magnetic stirring was selected as the optimum condition.

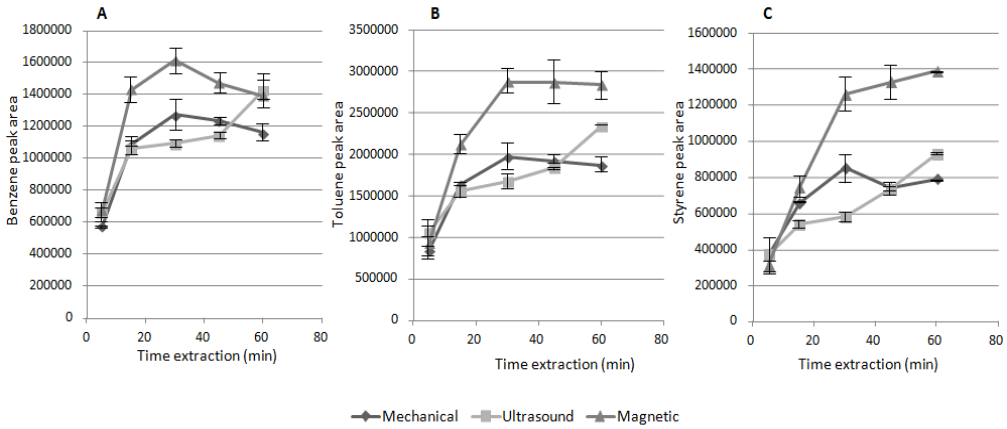


**Figure 1**. Effect of the stirring mode and time on the extraction of BTEX-S from waters. For simplicity only data obtained for benzene, toluene and styrene are shown.

### *3.2.3. Effect of the stirring rate*

The effect of stirring rate on the extraction efficiency was investigated from 0 to 900 rpm using 30 min as extraction time. As it can be seen in **Figure 2**, the extraction increased up to 900 rpm for all analytes. However, due to the morphology of the frit, the use of stirring rates higher than 900 rpm, induced a decreased in the extraction unit rotation. For this reason, 900 rpm was chosen as optimum for further studies.

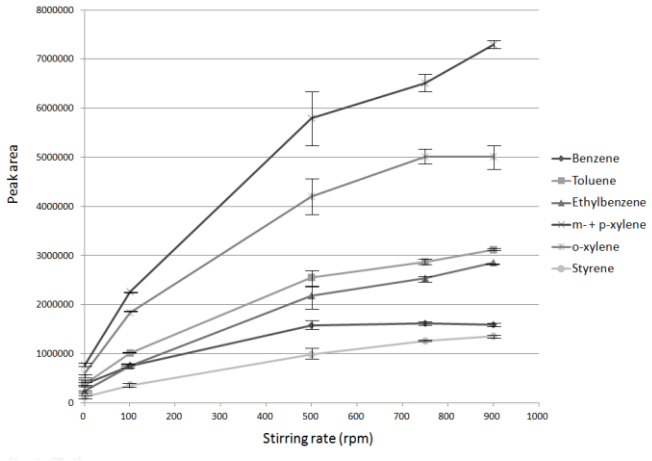


**Figure 2**. Effect of the stirring rate on the extraction of BTEX-S from waters.

### *3.2.4. Effect of sample volume*

The effect of sample volume on the analytes extraction was evaluated in a wide range, from 10 to 250 mL. The analytes extraction increased from 10 to 100 mL remaining almost constant for higher volumes. This effect may be ascribed to a lower stirring capacity of the frit when higher sample volume was employed. Thus, 100 mL was selected as optimal sample volume for ensuring studies.

## 3.3. Analytical figures of merit

The analytical features of merit of the proposed method are summarized in **Table 2**. The calibration curves for the analytes were constructed by using working aqueous standards prepared at controlled concentrations. An internal standard (*o*-xylene-$d_{10}$) at a concentration of 1 µg/L was used in order to improve the precision of the whole procedure including extraction and detection.

The precision of the procedure, expressed as relative standard deviation (% RSD), was studied using an aqueous standard at a concentration of 500 ng/L within and between days. As it can be seen in **Table 2**, the precision within days (intraday) ranged from 3.8% (toluene) to 8.2% (*m*- and *p*-xylene) while reproducibility between days (interdays) varied between 6.4% (*o*-xylene) and 14.8% (ethylbenzene).

The sensitivity of the method was evaluated according to the limit of detection (LOD), based on signal-to-noise ratio (S/N) of 3. LODs ranged from 18 ng/L (*o*-xylene) to 65 ng/L (benzene). Limits of quantitation, which were calculated using a S/N ratio of 10, were in the range from 60 ng/L (*o*-xylene) to 216 ng/L (benzene).

**Table 2**. Analytical figures of merit for the determination of the BTEX-S using the stir frit microextraction procedure.

| Analytes | m/z[a] | $R^2$ | LOD[b] (ng/L) | LOQ[c] (ng/L) | Linear range (µg/L) | Precision (%)[d] | | Preconcentration factor[e] | Log $K_{o/w}$[f] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Intraday (n=5) | Interday (n=3) | | |
| Benzene | 77, 78 | 0.999 | 65 | 216 | 0.2-100 | 7.3 | 11.6 | 157 | 2.06 |
| Toluene | 91, 92 | 0.999 | 34 | 113 | 0.1-100 | 3.8 | 9.7 | 465 | 2.61 |
| Ethylbenzene | 91, 106 | 0.999 | 22 | 73 | 0.07-100 | 5.0 | 14.8 | 998 | 3.09 |
| *m-, p*-xylene | 91 | 0.999 | 19 | 63 | 0.06-100 | 8.2 | 11.2 | 952 | 3.10 |
| *o*-xylene | 91, 106 | 0.999 | 18 | 60 | 0.06-100 | 4.2 | 6.4 | 946 | 30.2 |
| Styrene | 104 | 0.999 | 23 | 77 | 0.08-100 | 6.4 | 10.8 | 829 | 2.95 |

[a]Quantification ions (for details, see text).
[b]LOD, limit of detection.
[c]LOQ, limit of quantification.
[d]Precision expressed as relative standard deviation.
[e]Factors calculated comparing the calibrations graphs obtained with and without extraction. The theoretical maximum value is 1800.
[f]$K_{o/w}$, octanol water partition constant. Data obtained from SPARC on-line calculator.

Moreover, **Table 2** also summarizes the linear ranges obtained for each analyte. The preconcentration factors achieved with the stir frit microextraction have been calculated comparing the calibration graphs obtained with and without extraction. For this purpose, a direct calibration curve was built for each analyte using working standards with concentrations in the range from 0.5 to 20 mg/L. The direct calibration curve was

constructed adding 55 µL (total internal volume of the frit) of each standard to a dry frit which is subsequently introduced in a vial and analyzed following the headspace procedure. The slopes of both calibration graphs were compared and the resulting preconcentration factors (summarized also in **Table 2**) present an average value of 800.

The preconcentration factors as well as the octanol/water partition constants ($K_{o/w}$) obtained from SPARC-on line calculator [25], are also presented in **Table 2**. In the light of the results, there is a relationship between both parameters. In fact, if log PF is represented against the log $K_{o/w}$, a linear graph ($R > 0.99$) can be obtained. This aspect corroborates that the extraction of the analytes takes place due to the hydrophobic interaction with the PE frit. The high enrichment factors allow the reduction of the LODs of the method. Their impact in the results would be deeper if a final dilution of the analytes were not occurred. In fact, each frit is introduced in an empty vial (10 mL in volume) and therefore the analytes are diluted before their analysis. However, the reduction of the vial volume (introducing inert solids) did not lead to better results due probably to the lower effectiveness of the headspace mode in the thermal desorption of the analytes. Once optimized and analytically characterized, the proposed method was applied for the determination of the target analytes in water samples of different nature (well, tap and bottled water).

A recovery study was performed in order to evaluate the applicability of the proposed method to determine BTEX-S in waters. First of all, the samples were analyzed in order to find any potential presence of the analytes and later on they were spiked at two different concentration levels (0.5 and 5 µg/L). Each sample was analyzed by triplicate and the results are shown in **Table 3**. Taking into account the results obtained, it can be concluded that the extraction process was no affected by the different samples matrix. The recoveries were on average 94%. By way of an example, **Figure 3** shows the chromatogram obtained for a mineral water sample spiked with the analytes at a concentration of 500 ng/L.

**Table 3**. Recovery values obtained for the determination of BTEX-S in different water samples.

| Analytes | Tap | | Bottle | | Well I | | Well II | | Well III | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.5 µg/L | 5 µg/L | 0.5 µg/L | 5 µg/L | 0.5 µg/L | 5 µg/L | 0.5 µg/L | 5 µg/L | 0.5 µg/L | 5 µg/L |
| Benzene | 112 ± 8 | 95 ± 7 | 98 ± 7 | 85 ± 6 | 83 ± 6 | 95 ± 7 | 115 ± 8 | 84 ± 6 | 106 ± 8 | 95 ± 7 |
| Toluene | 79 ± 3 | 86 ± 3 | 96 ± 4 | 92 ± 3 | 61 ± 2 | 111 ± 4 | 80 ± 3 | 108 ± 4 | 116 ± 4 | 90 ± 3 |
| Ethylbenzene | 105 ± 5 | 90 ± 4 | 97 ± 5 | 84 ± 4 | 98 ± 5 | 121 ± 6 | 116 ± 6 | 123 ± 6 | 106 ± 5 | 91 ± 5 |
| *m-*, *p*-xylene | 96 ± 8 | 85 ± 7 | 98 ± 8 | 83 ± 7 | 102 ± 8 | 103 ± 8 | 113 ± 9 | 108 ± 9 | 98 ± 8 | 93 ± 8 |
| *o*-xylene | 97 ± 4 | 84 ± 4 | 96 ± 4 | 84 ± 4 | 98 ± 4 | 91 ± 4 | 111 ± 5 | 93 ± 4 | 96 ± 4 | 92 ± 4 |
| Styrene | 73 ± 5 | 75 ± 5 | 106 ± 7 | 83 ± 5 | 95 ± 6 | 95 ± 6 | 107 ± 7 | 94 ± 6 | 73 ± 5 | 95 ± 6 |

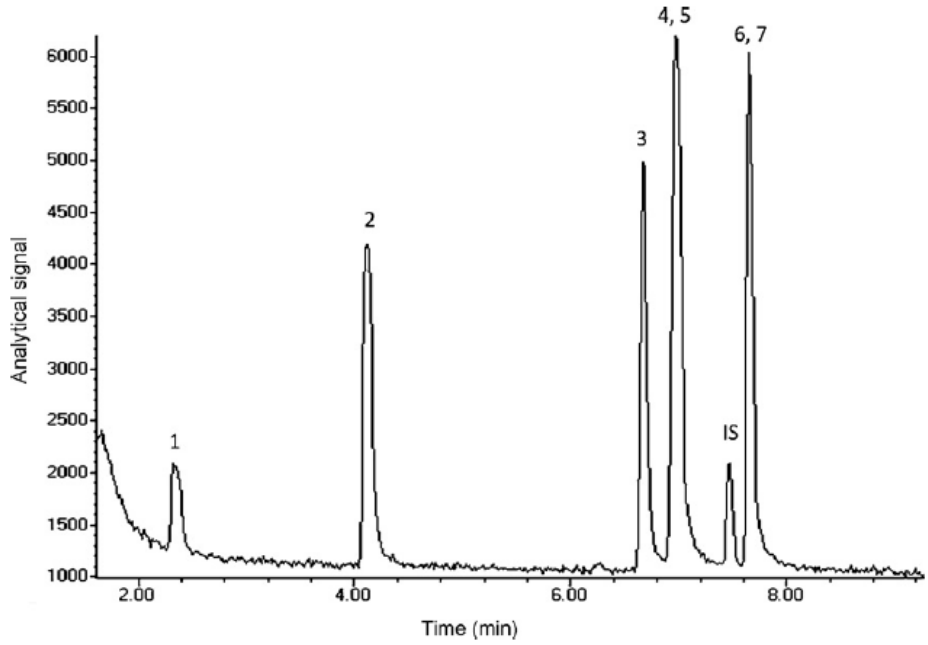


**Figure 3.** Chromatogram obtained for a mineral water sample spiked with the analytes at 500 ng/L, maintaining the internal standard at a concentration of 1 _g/L. (1) Benzene; (2) Toluene; (3) Ethylbenzene; (4) m-Xylene; (5) p-Xylene; (IS, internal standard) o-Xylene-$d_{10}$; (6) o-Xylene; (7) Styrene.

## 4. CONCLUSIONS

In the present article, a novel technique which combines the extraction capabilities of commercial frits and the well-known advantageous effect of stirring is described. For this purpose, each frit was pierced with an iron wire in order to allow the stirring of the device during the extraction procedure. This stirring facilitates the transference of the target analytes from the aqueous sample to the frit, providing high extraction efficiency.

The proposed extraction procedure has been systematically optimized taking into consideration the main instrumental and extraction variables and it has been evaluated using the determination of BTEX-S as the model analytical problem. The methodology allows the determination of BTEX-S in the nanogram per liter range with good precision. The combination of the stir frit with HS-GC/MS involves some advantages and disadvantages. First of all, it allows analyzing large sample volumes compared to the classic used in this technique. High enrichment factors, in the range from 157 to 998, can be obtained in only 30 min of extraction. Furthermore, the unit is quite simple and cheap.

Being objective, the procedure presents some shortcomings that should be overcome in further research. On the one hand, the variety of the frit material is limited which may reduce the applicability to a narrow range of analytes. In this case, some modifications, using new materials or modifying the commercial frits, will be considered in the near future. On the other hand, the reusability of the extraction frit is limited to 10 times in this application due to two facts, namely: the use of an unprotected iron wire as stirring element and the use of moderate-high temperatures and time of desorption. The first aspect involves the damage of the frit by inducing the appearance of oxide, while the second one affects to the porosity of the material. The use of a protected iron wire and chemical desorption (or the use of novel materials) may be the solution to both limitations.

Finally, the proposed method was compared to those employed for the GC/MS analysis of BTEX-S in water samples. The more representative examples [26–30] are presented in **Table**

**4** showing the RSD and LOD values. The proposal was comparable in terms of precision or sensitivity with other consolidated techniques. Although the stir frit microextraction provides high enrichment factors, the coupling with the headspace module involves a final dilution of the analytes. The use of thermal desorption units or the development of a chemical elution (coupled both to gas and liquid chromatography) are very promising alternatives for further investigations since they reduce the final dilution allowing the improvement of the sensitivity.

**Table 4.** Comparison of the performance of the proposed method with other extraction techniques coupled to gas chromatography/mass spectrometry for the determination of BTEX in water samples.

| Extraction Technique | Sample volume (mL) | RSD (%) | LOD (ng/L) | Reference |
|---|---|---|---|---|
| SFME-HS | 100 | > 3.8 | > 18 | This method |
| HS-SDME | 8 | > 3 | > 22 | [26] |
| HS-SPDE | 4 | > 8.8 | > 18 | [27] |
| HS-SPME-cryo trap | 15 | > 7.8 | > 0.01 | [28] |
| HS | 15 | > 4.1 | > 140 | [29] |
| HS-PTV | 5 | > 2.1 | > 10 | [30] |

[a]Acronyms: SFME-HS, stir frit microextraction headspace; HS-SDME, headspace single drop microextraction; HS-SPDE, headspace solid phase dynamic extraction; HS-SPME, headspace solid phase microextraction; HS, headspace.
[b]RSD, relative standard deviation.
[c]LOD, limit of detection.

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CAPÍTULO 2

# Micro-solid phase extraction based on oxidized single walled carbon nanohorns immobilized on a stir borosilicate disk: Application to the preconcentration of the endocrine disruptor benzophenone-3

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# Micro-solid phase extraction based on oxidized single walled carbon nanohorns immobilized on a stir borosilicate disk: Application to the preconcentration of the endocrine disruptor benzophenone-3

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A novel micro-solid phase extraction approach is presented. It consists of a borosilicate disk with oxidized single walled carbon nanohorns (o-SWNHs) immobilized in its pores. The o-SWNHs disk is placed in the screw of a portable drill that allows the extraction unit to be stirred inside the sample. The technique was applied to the extraction of benzophenone-3 from swimming pool water samples, followed by its determination with ultrahigh performance liquid chromatography with photodiode array detection. The variables affecting extraction were optimized, and the method was characterized in terms of linearity, limit of detection (0.16 μg/L) and precision (the relative standard deviation is lower than 11.9%). The enrichment factor is as high as 1379 and it involves an absolute recovery of 68.9%. Relative recoveries are close to 90%. The method is likely to represent a new approach towards microextraction of organic species using this nanomaterial.

***Keywords***: *Micro-solid phase extraction; Single walled carbon nanohorns; Stir borosilicate disk; Benzophenone-3; Swimming pool water.*

Graphical abstract

## 1. INTRODUCTION

Extraction and stirring integrated techniques have been in a continuous evolution [1] since the proposal of stir bar sorptive extraction (SBSE) [2] in 1999. However, few are the designs whose configurations can be adapted to perform on-site sampling [3]. SBSE shares the same general principles of solid phase microextraction (SPME) [4, 5] but it uses higher volume of extracting phases [6] which increases the overall extraction capacity. Off/on-site SBSE has been successfully accomplished by attaching the stir bar to a home-made miniature battery-operated portable electric stirrer [7]. This new configuration is very convenient to install, remove and replace the stir bar. Moreover, the coating loss by friction, which occurred frequently in conventional SBSE system, is avoided. Rotated-fiber and rotated-membrane samplers are also designed for field water sampling [8]. The commercially available fibers or membranes are coupled to a battery-operated drill which permits their stirring. Rotated-membranes use a higher volume of extracting phase and thus the extraction efficiency is improved. However, the rotated-fiber also provides some advantages, such as the easy introduction of the fiber into the gas chromatograph after the extraction. A more convenient fiber-retracted SPME device was also designed for field sampling [9].

Thin film microextraction (TFME) [10] was proposed as a new geometry for SPME in 2004. In TFME, a sheet of flat film with high surface area-to-volume ratio is used as extraction phase. With this configuration, the volume of the extraction phase increases while the thickness of the coating remains constant or it is even thinner. TFME has been investigated as sample preparation tool in many fields and it can easily conduct on-site sampling by using an electronic drill [11].

The exceptional properties of nanoparticles (NPs), specially their high superficial area and the variety of interactions, make them useful tools in extraction procedures. Single-walled carbon nanohorns (SWNHs) are a new type of carbon nanomaterial related to single-walled carbon nanotubes (SWCNTs) but with a conical shape [12]. Despite the potential of SWNHs,

few are the references which describe their use for analytical purposes [13-17] probably due to their scarce commercialization. To date, the extraction/stirring devices that use nanoparticles as extracting material are the so-called carbon nanotube assisted pseudo-stir bar solid/liquid microextraction [18], carbon nanotube-assisted pseudo-stir bar hollow fiber solid-liquid-phase microextraction [19] and magnetically confined hydrophobic nanoparticles [20, 21]. However, none of them can be easily adapted to perform on-site sampling or extraction.

Solid phase extraction disks based on the use of carbon nanomaterials have been proposed in the last years to extract organic compounds from large-volume environmental water samples. In these proposals, the disk is formed by passing a suspension of SWCNTs [22, 23] or multiwalled CNTs [24] though a polymeric filter where the NPs are retained. In this paper, the chemical immobilization of SWNHs into borosilicate disks is proposed to build a new extraction/stirring integrated technique. The mechanical resistance and easy functionalization of borosilicate disks, which has been recently evaluated by our research group [25], make them exceptional support in this type of extractions.

The novel proposal, that works under the micro-solid phase extraction principles, has been practically evaluated using the determination of benzophenone-3 (BP-3) in swimming pool water samples as model analytical problem. BP-3 is widely employed in sunscreens as it belongs to the group of ultraviolet (UV) filters, which are considered to be potential endocrine disrupting substances [26]. The increased use of these compounds would lead to environmental pollution as well as adverse health effects. For these reasons, UV-filters should be monitored in natural waters and efficiently sample preconcentration steps are required. To the best of our knowledge, this is the first method based on SWNHs and focused on the determination of BP-3 in water samples.

## 2. EXPERIMENTAL

### 2.1. Reagents and samples

All the reagents were of analytical grade or better. BP-3 (2-hydroxy-4-methoxybenzophenone) was purchased from Sigma–Aldrich (Madrid, Spain). Stock standard solution was prepared in acetonitrile (Sigma–Aldrich) at a concentration of 1 g/L and stored at 4ºC in the dark. Working standards were prepared on a daily basis by rigorous dilution of the stock in Milli-Q water (Millipore Corp., Madrid, Spain) or acetonitrile as required. Sodium chloride from Sigma-Aldrich was used in the optimization process.

Borosilicate filter disks (20 mm in diameter and pore size in the range of 16 – 40 μm), purchased from ROBU (Glasfilter-Geraete GmbH, Germany), were used as inert support for the immobilization of o-SWNHs. SWNHs, obtained from Carbonium S.r.l. (Padua, Italy), present purity better than 90 % and lengths in the range from 40 to 50 nm with diameters between 4 and 5 nm. The synthesis of borosilicate disks with immobilized SWNHs required the use of hydrogen peroxide, sulfuric acid, sodium hydroxide, 3-aminopropyltriethoxysilane (APTS), N-3(dimethylaminopropyl)-N-ethyl-carbodiimidehydrochloride (EDC·HCl), glutaraldehyde (GA), sodium borate, N,N'-dimethylformamide (DMF) and anhydrous acetone. All of these reagents were purchased from Sigma-Aldrich, acetone excepted (Panreac, Barcelona, Spain).

Different swimming pool water samples from Córdoba, Spain, were analyzed. The samples were collected in amber-glass bottles without headspace and stored in the dark at 4 ºC until their analysis. Filtration or pH adjustments were not required prior to the extraction process.

### 2.2. Apparatus

Chromatographic analyses were carried out on a Waters-AcquityTM Ultra Performance LC system (Waters Corp., Madrid, Spain) using an Acquity UPLC® BEH $C_{18}$ column (1.7 μm

particle size, 2.1 mm × 100 mm) maintained at 45 ºC. The mobile phase consisted of (A) water and (B) acetonitrile at a flow rate of 0.5 mL/min using a gradient elution program. The initial composition was fixed at 40% B, the percentage being increased to 60% in 5 min. The injection volume was 5 µL with partial loop with needle overfill mode. The separated analytes were determined using a PDA eλ (extended wavelength) Detector (Waters) at 290 nm. System control was achieved with Empower software.

A JEOL JSM 6300 scanning electron microscopy (Isaza, Alcobendas, Spain) was also used to obtain the micrographs of the borosilicate disk before and after the immobilization of the o-SWNHs. Micrographs were acquired in the Central Service for Research Support (SCAI) of the University of Córdoba.

### 2.3. Synthesis of the borosilicate disks with immobilized SWNHs

The functionalization of the borosilicate disk consists of several and well defined steps.

#### *2.3.1. Activation and functionalization of the borosilicate disk*

First of all, the borosilicate disk was activated by its immersion in a sulfuric acid: hydrogen peroxide (2:1, v/v) solution for 150 min at 100 ºC, the solution being renewed every 30 min in order to avoid losses due to evaporation. The activated disk was washed with Milli-Q water up to neutral pH and dried in an oven at 80 ºC for 12 h. In a second step, the borosilicate disk was introduced in NaOH 1M solution for 30 min under continuous stirring at 500 rpm. After that, the disk was washed with Milli-Q water up to neutral pH and dried in an oven at 80 ºC for 12 h. In a third step, amine groups were introduced in the disk by using a 2% v/v APTS solution prepared in anhydrous acetone. The solution, in close contact with the disk, was stirred at 500 rpm for 15 min and the disk was finally washed with Milli-Q water (10 min) and methanol (10 min) in order to eliminate the excess of APTS.

In a fourth step, the borosilicate disk was introduced in 10% v/v GA solution prepared in a 50 mM borate buffer at pH 9.0. After stirring the solution during 60 min at 500 rpm, the disk was washed with by Milli-Q water (10 min) and dried in an oven at 80 ºC for 12 h.

### *2.3.2. Oxidation of SWNHs*

First of all, 10 mg of pure SWNHs was accurately weighted in a glass vial. Subsequently, the nanomaterial was functionalized using microwave energy (800 W, 10 min) [27] introducing oxygenated functional groups on the nanoparticle surface, which could facilitate their dispersion in polar media. The functionalized solid was dispersed in 50 mL of Milli-Q water and then stirred for 60 min in an ultrasonic bath. The dispersion was centrifuged (J.P. Selecta, Barcelona, Spain) at 10000 rpm for 15 min to remove potential non-functionalized material, thus improving the reproducibility between dispersions. The dispersion was filtrated through a 0.5 µm polytetrafluorethylene tape (Miarco, Valencia, Spain) which was previously conditioned with methanol. The oxidized SWNHs (o-SWNHs) recovered were sonicated in methanol (20 mL) to remove them from the tape and they were finally dried in an oven at 40 ºC for 12 h. This procedure was repeated three times in order to obtain an amount of 10 mg of o-SWNHs.

### *2.3.3. Immobilization of o-SWNHs on the activated borosilicate disk*

The activated borosilicate disk was placed in a 25-mL glass beaker containing 20 mL of a EDC·HCl (0.01 g) solution in DMF and 10 mg of o-SWNHs. The disk was maintained in close contact with this dispersion for 3 h under a $N_2$ stream. Finally, the disk was washed with Milli-Q water and methanol to remove the non-immobilized o-SWNHs. The washing step was repeated twice.

**Figure 1** shows the micrographs obtained for the bare borosilicate disk (**Figure 1A**) and the borosilicate disk with the o-SWNHs immobilized on its pores (**Figure 1B**) at different magnifications. As it can be seen in **Figure 1B**, the surface of the borosilicate disk presents a rough aspect due to the immbolization of the o-SWNHs over the pores of the disk.

Attending to the size of the roughness of the disk, it corresponded with the diameter of the dahlias of the o-SWNHs. These results are in agreement with those obtained using hollow fiber as support [15].

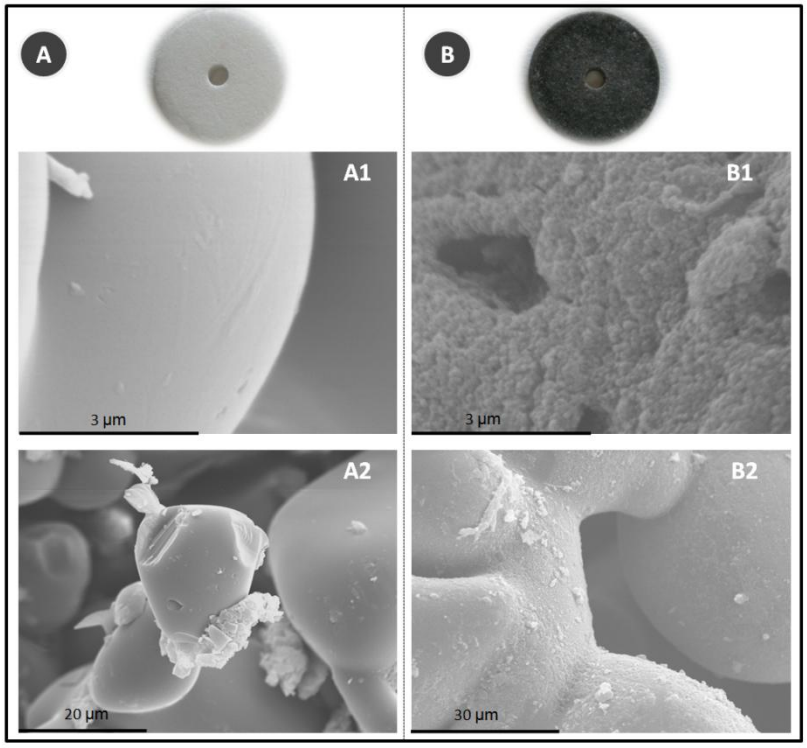


**Figure 1.** Scanning electron microscopy of borosilicate disk before (A1 x 16000, A2 x 1700) and after (B1 x 16000, B2 x 1300) the immobilization of the o-SWNHs on its pores.

The coupling of the o-SWNHs to the extraction device is depicted in **Figure 2**. The synthesized borosilicate disk with immobilized o-SWNHs (**Figure 2A**) was finally pierced with a screw and adapted to a rotating metallic axle (**Figure 2B**). The modified axle was finally integrated in a drill (**Figure 2C**) to make feasible its stirring (**Figure 2D**).


**Figure 2**. Pictures of the extraction device and process. (A) Blank (1) and SWNHs modified (2) disks; (B) Assembly of the disk to the metallic axle; (C) Coupling of the disk to the drill; (D) Extraction procedure.

## 2.4. Extraction procedure

The proposed extraction procedure is based on the principles of micro-solid phase extraction. For this reason, the extraction unit was previously conditioned with methanol (3 mL) and water (3 mL) and then introduced into glass beaker containing 200 mL of aqueous standard or sample, and stirred by means of a drill at 3000 rpm for 15 min to perform the extraction of the analyte. After the extraction, the o-SWNHs-modified disk enriched with the analyte is withdrawn and directly inserted on the lower section of a 20-mL syringe which is finally placed in a SPE vacuum manifold (Supelco, Bellefonte, PA). 5 mL of acetone are used to elute the analyte from the o-SWNHs-modified disk. To improve the sensitivity, the extract is evaporated to dryness under a nitrogen stream and the residue is finally

redisolved in 100 µL of acetonitrile and placed on a Total Recovery ® vial (Waters Corp.) for UPLC analysis.

Between extractions, the disk is rinsed with water and methanol to avoid potential carry-over.

## 3. RESULTS AND DISCUSSION

### 3.1. Optimization of the extraction conditions

Different variables, which are summarized in **Table 1**, may affect the efficiency of the extraction procedure and therefore their effects on the analyte extraction were considered in depth in the optimization process. **Table 1** also reflects their initial values, the interval studied and the optimum values. As it is a novel extraction procedure, a univariate approach was selected to study the effect of each single variable in the extraction process.

An aqueous standard solution containing benzophenone-3 at a concentration of 50 µg/L was used in these studies.

**Table 1**. List of the variables involved in the SWNHs-modified stirring disk extraction process.

| Variable | Initial value | Interval studied | Optimum value |
|---|---|---|---|
| pH | 6.8 | 3 – 10 | 3-7 |
| Ionic strength (g/L NaCl) | 0 | 0 - 200 | 0 |
| Sample volume (mL) | 200 | 50 - 1000 | 200 |
| Extraction depth (cm) | 4 | 2 - 4 | 4 |
| Extraction time (min) | 10 | 5 - 60 | 15 |
| Elution solvent (mL) | Acetonitrile | Acetonitrile, methanol, acetone, ethanol | Acetone |
| Elution volume (mL) | 1 | 1-10 | 5 |

### *3.1.1. Effect of sample pH and ionic strength*

The pH value of the sample usually plays a key role on the extraction performance since it defines the charge of the analyte (BP-3; pKa=7.5) and even the superficial charge of the sorbent. The sample pH was studied in the range from 3 to 10 (data not shown) and the results showed that the highest enrichment factors (EFs) were obtained in the range from 3 to 7 with negligible differences among them.

The influence of the ionic strength was evaluated using sodium chloride (NaCl) as model electrolyte varying its concentration in the sample in the range from 0 to 200 g/L. The ionic strength enhances the extraction efficiency of the analyte (by a salting-out effect) but negatively affects the precision of the measurements (probably due to the viscosity increase of the media). For this reason, no extra ionic strength was added in further analyses.

### *3.1.2. Effect of the sample volume*

The effect of the sample volume on the BP-3 extraction was investigated from 50 to 1000 mL. As it can be seen in **Figure 3** the analyte extraction increased from 50 to 200 mL remaining almost constant for higher volumes. In order to asses that the sorbent capacity was not overloaded when high samples volumes are used, an additional assay was performed. In this sense, 500 mL of three standard solutions containing the analyte at different concentrations (20, 50 and 100 µg/L) were extracted by the proposed unit. The results showed an increased of the signal with the analytes concentration which clearly demonstrates that the sorbent capacity was not overfilled.

Therefore, the results of **Figure 3** can be explained taking into consideration the stirring capacity of the unit which is able to homogenize a defined sample volume around it. In this sense, in the interval from 200 mL to 1000 mL the extraction is almost independent of the sample volume which opens a door to on-site extraction.

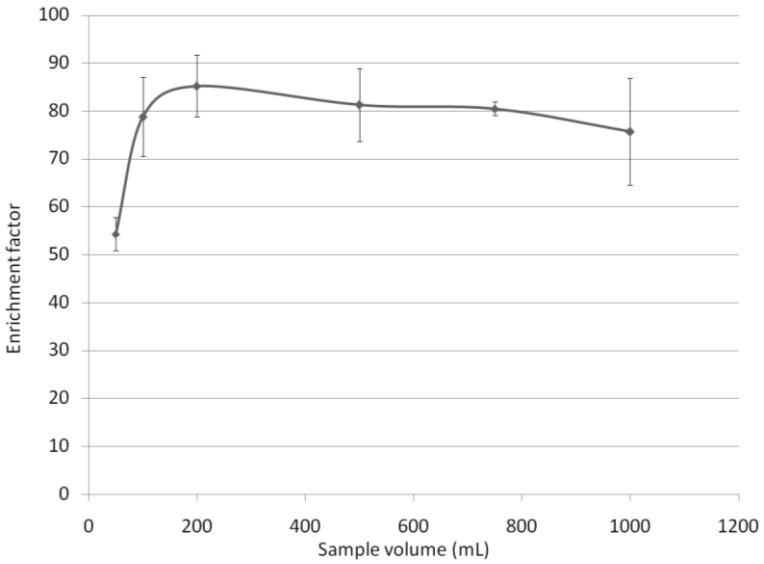


**Figure 3.** Effect of the sample volume on the enrichment factors of the BP-3.

### *3.1.3. Effect of the extraction depth*

Extraction depth is the immersion distance of the o-SWNHs-modified borosilicate disk below the sample surface. This variable was evaluated in the range from 2 to 4 cm (data not shown) since larger depths require longer metallic axes which are not stable enough. Although the average signal is not affected by the extraction depth, the precision becomes worse when the shorter depth is employed. Therefore, sampling depth was fixed at 4 cm for further studies.

### *3.1.4. Effect of the extraction time*

The influence of the extraction time (or stirring time) was investigated in the range from 5 to 60 min, and the results are depicted in **Figure 4**. The extraction increases markedly and

almost linearly with the time up to 15 min remaining almost constant for larger times. Therefore, 15 min were fixed for further studies.

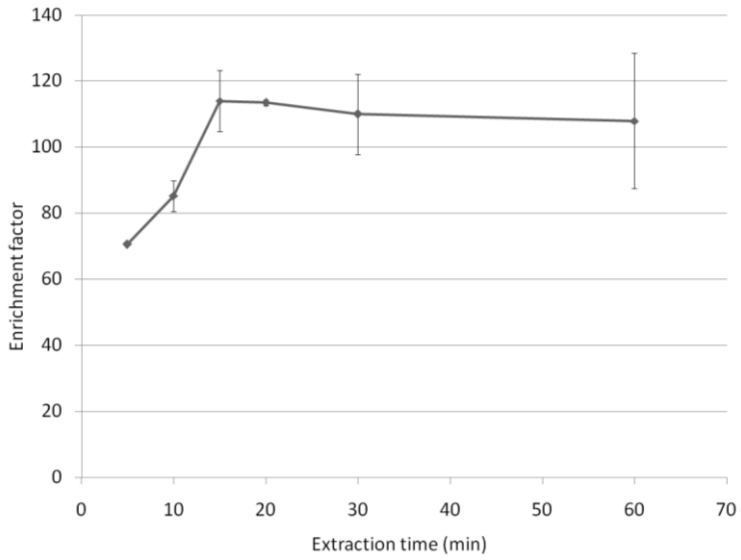


**Figure 4.** Effect of the extraction time on the enrichment factors of the BP-3.

### *3.1.5. Elution step*

Once the analyte has been conveniently extracted, it should be eluted for the subsequent chromatographic analysis. For this purpose, the o-SWNHs-modified disk containing the retained analyte, previously dried, is placed into a 20-mL syringe lower section, which is located in a SPE vacuum manifold. The elution is performed passing an appropriate solvent through the disk. Different organic solvents were tested for this purpose, namely: acetonitrile, methanol, acetone and ethanol. As it can be seen in **Figure 5**, acetone provided the best results in terms of sensitivity (higher areas) and precision (lower relative standard deviation) and it was selected as eluent. Moreover, acetone is more economical than the other solvents. The elution volume was studied taking into account that the final

extract will be evaporated under a $N_2$ stream for sensitivity enhancement. This step is possible since no losses by evaporation are observed for the target analyte. Therefore, the volume should be higher enough to provide an efficient elution of the analyte but lower enough to allow a rapid evaporation. The elution volume was studied in the range from 1 to 10 mL (data not shown), 5 mL of acetone being selected as a compromise between both effects. Finally, the extract was redisolved in 100 µL of acetonitrile for compatibility with the instrumental analysis.

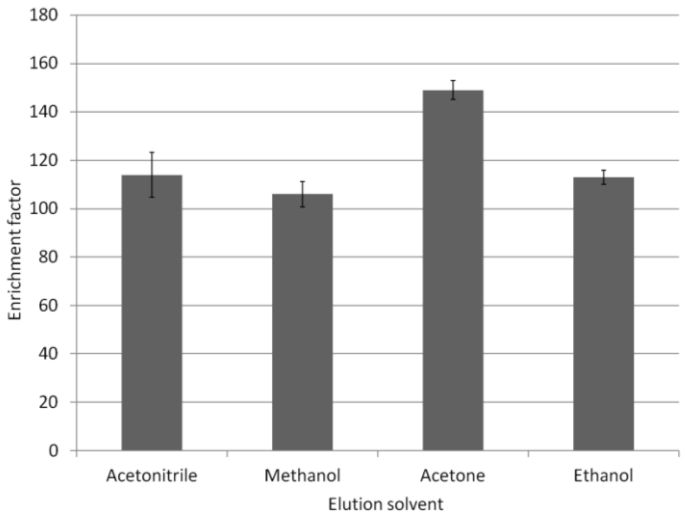


**Figure 5.** Effect of the elution solvent on the enrichment factors of the BP-3.

### 3.2. Analytical figures of merit

The analytical figures of merit of the proposed method are summarized in **Table 2.** The calibration curve for benzophenone-3 was constructed by using nine working aqueous standards prepared at controlled concentrations which were subjected to the optimized

extraction procedure. The method was characterized on the basis of its linearity, sensitivity, precision and accuracy.

The sensitivity of the method was evaluated according to the limit of detection (LOD) and quantification (LOQ). LOD, calculated using a signal-to-noise ratio (S/N) of 3, was 0.16 µg/L while the LOQ, calculated using a S/N of 10, was 0.51 µg/L. The linearity was maintained up to 100 µg/L.

Within-disks precision was evaluated in terms of relative standard deviation (RSD) at two different concentration levels: 5 µg/L and a concentration closer to the limit of quantification. As it can be seen in **Table 2,** within-disks repeatability was 8.9% and 11.9% at 5 µg/L and LOQ, respectively. Moreover, disk-to-disk precision was evaluated at 5 µg/L using three independents disks, and it resulted to be 11.8% (n=3).

**Table 2.** Analytical figures of merit of the proposed method for the determination of BP-3 in swimming pool water sample.

| Analyte | LOD[a] (µg/L) | LOQ[b] (µg/L) | Precision[c] (%) | | | EF[d] | AER[e] (%) |
|---|---|---|---|---|---|---|---|
| | | | Repeatability (within-disks) | | Disk-to-disk reproducibility (between-disks) | | |
| | | | At 5 µg/L (n=5) | At LOQ[b] (n=5) | At 5 µg/L (n=3) | | |
| BP-3 | 0.16 | 0.51 | 8.9 | 11.9 | 11.8 | 1379 | 68.9 |

[a]LOD, limit of detection, calculated for a S/N of 3.
[b]LOQ, limit of quantification, calculated for a S/N of 10.
[c]Precision expressed as relative standard deviation.
[d]EF, enrichment factor. The theoretical maximum value is 2000.
[e]AER, absolute extraction recovery.

The extraction performance of the proposed technique was evaluated by means of the enrichment factor (EF) and absolute extraction recovery (AER). EF, which was calculated by

comparing the calibration graphs before and after the extraction process, is 1379 which corresponds to an AER of 68.9%.

Finally, the accuracy of the method was evaluated by means of a relative recovery study using four independent swimming pool water samples. First of all, the samples were analyzed in order to find any potential presence of the analyte. No positives samples were obtained which can be ascribed to two complementary facts: the low BP-3 contamination load in these waters and/or to the partial BP-3 degradation by chlorination agents employed in the water treatment. Both factors may reduce the concentration of the parent compound. Therefore, samples were spiked at 5 µg/L concentration level and analyzed by the proposed method. From the results listed on **Table 3**, relative recoveries presented an average value of 90%, fulfilling the 70-130% recovery criterion [28], The results show the potential of the proposed stir o-SWNHs-modified disk for the extraction of BP-3 from swimming pool waters.

**Table 3.** Relative recovery study performed on swimming pool water samples spiked with BP-3 at a concentration of 5 µg/L.

| Analyte | Swimming pool water samples (R% ± SD) | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| BP-3 | 110 ± 8 | 78 ± 9 | 80 ± 9 | 92 ± 8 |

R% extraction recovery, SD standard deviation (n=3).

## 4. CONCLUSIONS

In the present article, a novel micro-solid phase extraction technique, that integrates extraction and stirring in the same device, is presented. For this purpose, o-SWNHs are immobilized on a previously activated borosilicate disk. Once synthesized, the o-SWNHs-

disk is pierced with a screw and adapted to a rotating metallic axle. The modified axle is finally integrated in a drill to make feasible its stirring. The novel coating material is used for the extraction of BP-3 in swimming pool waters.

The new extraction device is able to homogenize a defined sample volume around it. For this reason, the extraction is almost independent of the sample volume which opens a door to on-site extractions. Unfortunately, a critical comparison between off-site and on-site extraction mode was not possible due to the absence of positive samples.

After a careful optimization of each variables affecting to the analytical procedure, the sorbent capacity of the SWNHs-modified disk was tested in different swimming pool water samples, obtaining excellent results both in recovery values and in terms of sensitivity (in the low microgram per liter range). Moreover, satisfactory precision was obtained intra-disk and among different synthesized disks. The new proposal provides extremely high enrichment factors, over 1300. Furthermore, the extraction unit is reusable since no carry-over between samples was observed.

Being critical, the procedure presents some shortcomings that should be overcome in the near future. The synthesis of the borosilicate disks with immobilized SWNHs is time-consuming. The weakness of the technique lies on the lack of automation which may limit its applicability in routine analysis.

Taking into account the excellent sorptive properties of carbon nanohorns, further research should be aimed at evaluating their usefulness in other microextraction techniques. In this sense, the application field of these carbon nanoparticles could be extended to other matrices or analytes of different polarity by including different functional groups on their surface. Moreover, borosilicate disks are useful extraction materials because of their good sample-to-adsorbent contact surface due to their high porosity. Indeed, the device presents great potential in the microextraction context due to the versatility of the disk derivatization.

Finally, **Table 4** compares the proposed method with other methodologies for the determination of BP-3 in water samples [29-51]. These approaches cover a wide range of extractions as well as instrumental techniques. According to the results, the new proposal provides one of the highest enrichment factors. Moreover, the extraction time is considerably shorter. In addition to this, precision levels are comparable with those of the other approaches.

The sensitivity of the overall methodology could be considered the weak factor in the comparison with other methodologies. However, this sensitivity depends on the inherent sensitivity of the instrumental technique and the efficiency of the extraction procedure, among other factors. The latter one cannot be considered critical in this application attending to the obtained enrichment factor (1379). Therefore, the sensitivity could be enhanced using another instrumental technique like GC/MS or LC/MS. In fact, a similar detection limit is obtained in comparison with other published methodologies [44-48, 51] based on UV or DAD detection.

**Table 4.** Comparison of the proposed extraction procedure with other published methods for the extraction of BP-3 from waters.

| Extraction technique[a] | Aqueous matrix | Sample volume (mL) | Extractant[b] | Instrumental technique[c] | Extraction time (min) | LOD[d] (µg/L) | EFs[f] | Relative recovery (%) | Precision (%RSD[g]) | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| DI-SPME<br>HS-SPME | Swimming pool and bathing waters | 5 | 100-µm PA<br>85-µm PDMS | GC-MS | 45 | 1.17-4.10 (LOQ[e]) | -- | 91-99 | <8.9 | [29] |
| SPE | Natural water | 500 | 500 mg $C_{18}$ | GC- MS | -- | 0.0014 | -- | 95-97 | <5 | [30] |
| SPE | Tap water and clean water | 200 | 500 mg $C_{18}$ | LC-DAD<br>GC-MS | -- | 0.014<br>0.0074 | -- | 74 | <8.6 | [31] |
| SPE | River | 1000 | 60 mg Oasis MCX | CE-ESI-MS | -- | 150 | -- | -- | <10.8 | [32] |
| CPE | Bathing waters | 10 | Triton X114 | LC-UV DAD<br>GC-MS | 30 | 0.45<br>0.0062 | 67.7 | 98.8-99 | <4.3<br><5.6 | [33] |
| SBSE | Lake, river and treated wastewater | 20 | 22-mg PDMS | TD-GC-MS | 180 | 0.011 | -- | 92-112 | <15 | [34] |
| SBSE-LD | Wastewater and river | 50 | PDMS | UPLC-ESI-MS/MS | 120 | -- | 212 | 59-67 | <26 | [35] |
| SBSE | Lake | 250 | PDMS | DART-MS | 240<br>1200 | 0.02 | -- | -- | -- | [36] |
| SBSE-LD | Sea | 10 | PDMS | LC-APCI-MS/MS | 180 | 0.08 | -- | 83 | <11 | [37] |
| SBSE-LD | Sea, river and wastewater | 50 | 24-µL PDMS | HPLC-APCI-MS/MS | 300 | 0.0009 | -- | 64 | <7 | [38] |
| SBSE-LD | Urban wastewater treatment plants | 50 | PDMS | LC-MS/MS | 300 | 0.0015 | -- | 61 | <6 | [39] |
| (DI) plunger in-needle SPME | River | 7 | Graphene | GC/MS | 40 | 0.0007 | 2558 | 102 | 5.6 | [40] |
| MALLE | Surface water and wastewater | 15 | 100 µL propanol | LC-APCI-MS/MS | 120 | 0.0008 | -- | -- | <17 | [41] |

| Extraction technique[a] | Aqueous matrix | Sample volume (mL) | Extractant[b] | Instrumental technique[c] | Extraction time (min) | LOD[d] (µg/L) | EFs[f] | Relative recovery (%) | Precision (%RSD[g]) | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| SD | Surface and sewage water | 100 | 12 µL silicone | GC-MS | 840 | 0.04 (LOQ[e]) | -- | 75-108 | <9.9 | [42] |
| dSPE | Tap water, river, sea | 75 | MNPs | GC-MS | 9 | 0.0002 | 748 | 80-106 | 5.6 | [43] |
| IL-HF-LPME | River | 10 | 7 µL [HMIM][FAP] | HPLC-UV | 50 | 0.20 | 216 | 98.1-104.9 | <1.1 | [44] |
| IL-SDME | Surface water | 20 | 10 µL [$C_6$MIM][$PF_6$] | LC-UV | 37 | 0.11 | 98 | 98 | <2.8 | [45] |
| DLLME | Lake | 20 | 40 µL 1-octanol | HPLC-UV | 20 | 0.8 | 107 | 94.2 | 4.6 | [46] |
| IL-USA-DLLME | Tap water, river, swimming pool | 10 | 20 µL [HMIM][FAP] | HPLC-UV | 8 | 0.5 | 354 | 105-114 | 5.5 | [47] |
| UDSA-IL-DLLME | Environmental water | 5 | 40 µL [C8MIM][PF6] | UPLC-UV | 6 | 0.23 | 183 | 102-120 | <1.3 | [48] |
| VADLLME | River and reservoir water | 10 | 40 µL tetrachloroethene | GC-MS | 3 | 0.017 | 152 | 76.1-110.4 | <6.1 | [49] |
| KW-LPME | Swimming pool | 20 | 8 µL 25% (v/v) PCE:1-octanol | HPLC-UV | 30 | 0.02 | 366 | 78.4 | <10.2 | [50] |
| TD-IL-DLPME | Tap water and swimming pool | 10 | 20 µL [HMIM][FAP] | HPLC-UV | 20 | 0.8 | 240-350 | 90-100 | <8.1 | [51] |
| Stir SWNHs-borosilicate disk (µ-SPE) | Swimming pool | 200 | SWNHs | UPLC-DAD | 15 | 0.16 | 1379 | 77.9-109.9 | <11.9 | This method |

[a]Acronyms: DI-SPME, direct immersion solid phase microextraction; HS-SPME, headspace solid phase microextraction; SPE, solid phase extraction; CPE, cloud point extraction; SBSE, stir bar sorptive extraction; SBSE-LD stir bar sorptive extraction-liquid desorption; MALLE, membrane-assited liquid-liquid extraction; SD, silicone discs; dSPE dispersive solid phase extraction; IL-HF-LPME, ionic-liquid hollow fiber liquid phase microextraction; IL-SDME, ionic liquid single drop microextraction;DLLE, dispersive liquid-liquid microextraction; IL-USA-DLLME, ionic-liquid ultrasound-assisted dispersive liquid-liquid microextraction; UDSA-IL-DLLME, up-and-down shaker-assisted ionic-liquid-based dispersive liquid–liquid microextraction; VADLLME, vortex-assisted liquid-liquid microextraction; KW-LPME, knitting wool liquid phase microextraction;TD-IL-DLPME, temperature-controlled ionic liquid dispersive liquid-phase microextraction; SWNHs, single walled nanohorns.
[b]Acronyms: PA, polyacrylate; PDMS, polydimethylsiloxane; MCX, mixed-mode cation-exchange; MNPs, magnetic nanoparticles; [HMIM][FAP], 1-hexyl-3-methylimidazolium tris(pentafluoroethyl)trifluorophosphate); [$C_6$MIM][$PF_6$], 1-hexyl-3-methylimidazolium hexafluorophosphate; [$C_8$MIM][$PF_6$]; PCE, perchloroethylene.
[c]Acronyms: GC, gas chromatography; MS, mass spectrometry; LC, liquid chromatography; DAD, diode array detection; CE, capillary electrophoresis; ESI, electrospray ionization; UV, ultraviolet;TD, thermal desorption; UPLC, ultra performance liquid chromatography; DART, direct analysis in real-time; APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionisation.
[d]LOD, limit of detection.
[e]LOQ, limit of quantification.
[f]EF, enrichment factor.
[g]RSD, relative standard deviation.

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CAPÍTULO 3

# Solid phase microextraction under the thin film format

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# Solid-phase microextraction under the thin film format

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Thin film microextraction (TFME) is considered a type of solid-phase microextraction (SPME). There has been a growing interest in TFME as a novel sample preparation technique, which was originally introduced to address the limiting uptake rate and capacity sometimes observed with fiber microextraction. The inherent properties of TFME technique such as the excellent sample clean-up and the larger surface to volume ratio, enhance the sensitivity and the extraction rates. This e-book article is mainly focused on the fundamental principles behind and in the diverse existing TFME configurations, paying particular attention to cotter pin supported format, copper mesh holder and 96-blade format.

***Keywords:*** *Autosampler; Field sampling; In-vivo sampling; Membrane-based TFME; Microextraction; On-site extraction; Sample preparation; Solid phase microextraction (SPME); Sorptive tape extraction; Stirring integrated TFME; Thin film microextraction (TFME); 96-blade (thin film) SPME.*

## 1. INTRODUCTION

Solid-phase microextraction (SPME) is a sample preparation technique developed by Pawliszyn in the early 1990s which integrates sample clean-up, analyte isolation, and pre-concentration in a single step [1]. This technique appeared to address the need for a rapid and solvent-free extraction technique. SPME is based on the partitioning of the analytes between the sample and the extraction phase, which is typically immobilized on a fiber support. For direct immersion, the amount of analyte extracted when equilibrium conditions are reached ($n_e$) by SPME can be described as [2]:

$$n_e = \frac{K_{es} V_e V_s C_0}{K_{es} V_e + V_s} \quad \text{(Equation 1)}$$

Where $K_{es}$ is the distribution constant between the extraction phase and the sample matrix, $V_e$ represents the sorbent phase volume, $V_s$ is the sample volume and $C_0$ is the initial concentration of a analyte in the sample matrix. Since in the majority of the extractions the sample volume is higher than the extraction phase, this equation can be simplified to:

$$n_e = K_{es} V_e C_0 \quad \text{(Equation 2)}$$

According to **Equation 2**, the mass of analyte extracted depends on the sorbent phase volume ($V_e$). There are two possible strategies to increase the extraction phase volume: (i) make greater in size the thickness of the coating or (ii) expand the surface area. However, when thicker coatings are employed larger times are needed to reach the equilibrium ($t_e$), which gives rise low sample throughput because the overall rate of extraction is controlled by the diffusion from the bulk solution through the boundary layer to the extraction coating (**Equation 3**):

$$t_e = t_{95\%} = 3\frac{\delta K_{es}(b-a)}{D} \quad \text{(Equation 3)}$$

where *(b-a)* is the thickness of the coating, *D* represents the diffusion coefficient and $\delta$ is the thickness of the boundary layer. Based on **Equation 3** [3], the thinner the thickness of the sorbent phase is, the shorter the time required to reach the extraction equilibrium it would be.

On the other hand, large surface area accelerates the initial rate of extraction as it is shown in **Equation 4** [3]. As a consequence, a thin film with a large surface area-to-volume ratio ensues in an improvement of the extraction efficiency without sacrifice the extraction time assuming the same conditions.

$$\frac{dn}{dt} = \left(\frac{DA}{\delta}\right)C_o \quad \text{(Equation 4)}$$

Where *(n)* is the amount of analyte extracted over the sampling time *(t)* and *(A)* is the surface area of the extraction phase. On the basis of the previous discussion, the superficial area of a SPME phase directly affects to the extraction kinetics and this theoretical fact has led to the development of thin film microextraction (TFME) by Prof. Pawliszyn group in 2003 [3]. According to the inventors group, TFME can be divided in two different modes depending on the use of the membranes or brushes as extracting phase [4].

## 2. THIN FILM MICROEXTRACTION UNDER THE MEMBRANE CONFIGURATION

Membrane-based TFME makes use of a thin membrane of polydimethylsiloxane (PDMS), the classical SPME coating, as extraction phase. In the classical configuration the membrane is

attached to a deactivated stainless steel rod and it is deployed, in a flag shape, to exploit all its potential area during extraction (see **Figure 1**). After the extraction, which can be accomplished both in the direct immersion and headspace mode, the membrane is coiled around the rod which is finally introduced in a glass liner for gas chromatography/mass spectrometry (GC/MS) analysis.

The first approach of TFME, which was directly towards the analysis of several polycyclic aromatic hydrocarbons (PAHs) from water samples, demonstrated the superior features of TFME over classical SPME [3]. In summary, TFME presents a higher extraction rate and capacity owing to the larger area and higher volume of extractant phase (at least 4 times) in the membrane format over the fiber shape. In fact, while a typical 100 μm PDMS fiber presents an area of 10 $mm^2$, a 1 cm x 1 cm membrane sheet presents a surface area of 200 $mm^2$ (two faces of 100 $mm^2$ each one) due to its planar structure.

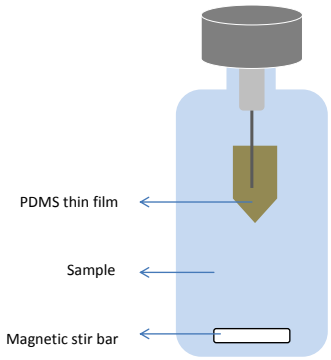


**Figure 1.** Cotter pin supported format of TFME.

The stirring of the sample during extraction is critical to achieve an acceptable extraction rate. In fact, as it can be deduced form **Equation 3**, the time required to reach the extraction equilibrium depends on the thickness of the boundary layer which may be controlled with the efficient stirring of the sample solution. This aspect, applicable to all the

microextraction techniques, plays a key role on those extraction/stirring integrated techniques. Stir bar sorptive extraction, developed by Prof. Sandra group in 1999 [5], is the clear paradigm in this context and presents a clear similarity with TFME since a PDMS phase coated on a glass jacketed iron bar is used as extractant. Qin *et al.* compared TFME and SBSE in terms of kinetics and efficiency using some PAHs as model analytes [6]. However, the different stirring performance of both techniques (SBSE integrates extraction and stirring in the same device whereas, as it is shown in **Figure 1**, classical TFME is performed with an external magnetic bar) limits their direct comparison.

To normalize both performances, the researchers modified the TFME procedure integrating the thin film in an electric drill, which produces the stirring of the extraction phase rather than the solution. Moreover, the researchers developed a new holder to attach the thin film, avoiding their bending during the stirring. In the light of the extraction rate profiles obtained for different PAHs, TFME resulted to be faster than SBSE. This observed behavior can be explained taking into account **Equations 3** and **4**. On the one hand, $t_e$ increases with the thickness of the extraction phase which is higher in SBSE than in TFME. On the other hand, the initial extraction rate increases with the extractant area which is larger in TFME. Additionally, stirring integrated TFME opens a door to on-site extractions (**Figure 2**) which is quite interesting in environmental applications since the target analytes usually are more stable in the extractant phase than in the original sample.

**Figure 2.** On-site sampling. Reproduced with permission of Elsevier from Reference [7].

This aspect has been studied in depth by the same research group of the first development using some PAHs as model analyte. In this work they developed a new configuration based on a copper mesh holder (**Figure 3**) in order to protect the thin film during the extraction and to make easier the transference of the film to the glass liner for chromatographic analysis [7].

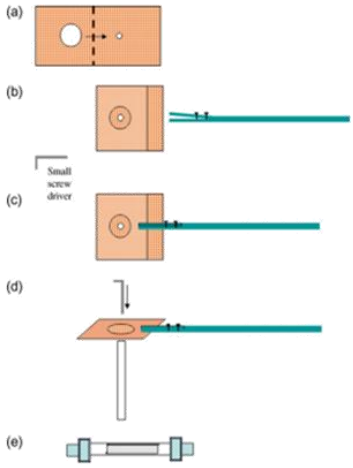


**Figure 3.** Thin film copper mesh. (a) Fold (b, c) fix on a rod (d) push into a liner (e) seal the liner for transportation. Reproduced with permission of Elsevier from Reference [7].

TFME can be also applied in field sampling in order to measure the time-weighted average concentration of target pollutants in environmental specimens. This approach has been evaluated for the monitoring of PAHs in river water [8] using a thin film of PDMS (127 μm in thickness) as sorptive phase. The thin film (see **Figure 4**) is cut into a house-shape format that consists of a square of 2 cm x 2 cm with an additional triangular section of 1 cm in height. The thin film is attached to a stainless steel wire that plays two roles: (i) it serves as support of the thin film and (ii) allows its introduction on a GC liner (see **Figure 4**) for final analysis. For field sampling, the thin film is introduced in a copper cage to protect the sorptive phase from degradation and contamination of algae. The sampling is performed for a defined time and after sampling the thin film is recovered and stored in a sealed vial

until analysis. Before analysis, the thin film is washed with water, rolled up and introduced in a glass inlet for GC/MS analysis. The inherent irreproducibility of field sampling, due to the different variables that may affect the extraction, is overcome using an internal standard (deuterated analytes) impregnating the membrane. The internal standard losses during the extraction provided information about the sampling conditions (time, volume of sample), normalizing their effect.

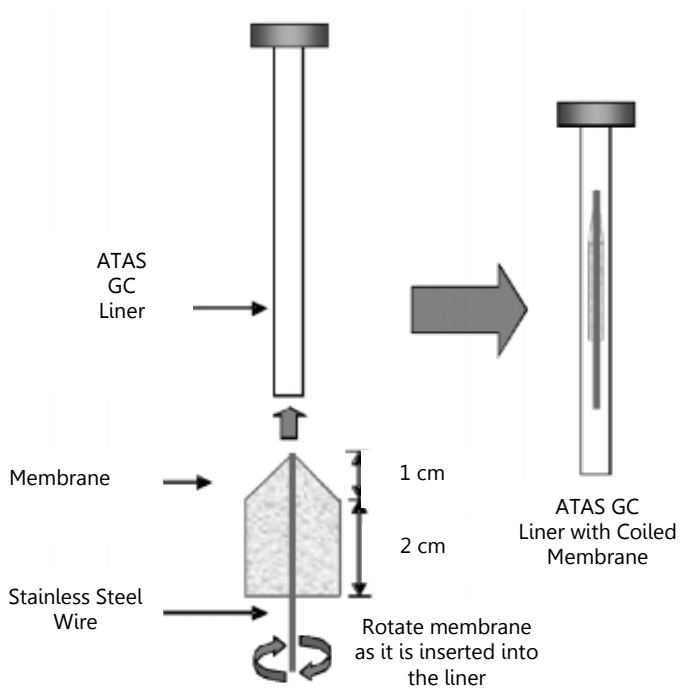


**Figure 4**. PDMS thin-film configuration and its coupling into the ATAS GC liners for analysis. Reproduced with permission of Oxford Journals from Reference [8].

Although the first developments of TFME were focused on the extraction of liquid samples, TFME can be applied for the extraction of gaseous samples which is quite interesting in

environmental analysis. In this sense, Eom *et al.* employed the technique for the monitoring of ambient air for the detection of biomarkers of the presence of *Cimex lectularius* [9]. In the extraction procedure, the thin film is located in the back-side of a small electric fan which circulates air in order to increase the sampled volume. As in any gas-solid partitioning process, the extraction temperature plays a key role on the sorption of the analytes. The reduction of the sorptive phase temperature should increase the extraction efficiency. In view of this, Jiang and Pawliszyn have recently proposed the use of cooled membranes in gas sampling [10] using a special extraction device (**Figure 5**). The device allows the continuous flow of sample gas through the sorptive phase and the membrane cooling by means of an electric fan and thermoelectric cooler, respectively. In addition, the device permits the direct introduction of the membrane after the extraction in a thermal desorption tube for GC/MS analysis avoiding losses of the target analytes.

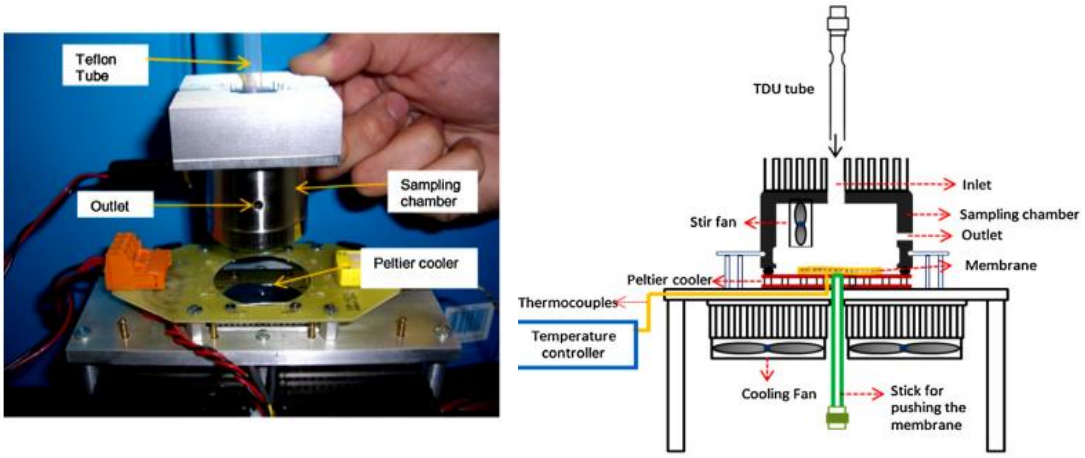


**Figure 5**. Configuration of cooled membrane device. Reproduced with permission of Elsevier from Reference [10].

The great versatility of the technique allows its application to process solid samples. In this context, sorptive tape extraction (STE) was proposed by Prof. Sandra´s group in 2006 as a microextraction technique adapted to the sampling of the surface of a given system [11]. As

TFME, STE is based on the use of a flexible thin film of PDMS phase which is attached to the sample acting as chemical trap which retains the analytes on the basis of its sample/PDMS partition coefficient. In its first proposal, STE was applied for the qualitative and quantitative characterization of skin sebum. A thin film of PDMS is attached to a defined area of the skin with a plaster and the sampling/extraction is developed in a defined time, usually 15 min. The extracted analytes can be thermally desorbed or chemical eluted with acetone prior to their final determination by GC/MS. Although the thickness (0.5 mm) and length (15 mm) of the PDMS are fixed, the width of the film is different depending on the final procedure. In this sense, the width is 4 mm and 12 mm for thermal desorption and chemical elution, respectively, involving a different sampled area in each application. STE provided comparable results than classical techniques employed in sebum analysis. It is simpler, more precise and present a wide application field that may comprise not only the sebum characterization (different compounds and concentrations) but also the in-vivo analysis of the efficiency of skin treatment that is of outstanding importance in the clinical context.

The same research group has extended the application of STE to the analysis of biological solid matrices which can be directly or indirectly sampled, depending whether the thin tape is located on the surface of the material in direct contact (DC)-STE, or in close contact with its headspace (HS-STE) [12]. Two different extraction vessels can be employed depending on the type of matrix to be analyzed using in both cases a STE holder, a stainless steel piece which acts as support of the thin tape. For in-vivo sampling of plant leaves, a vial with four inlets (as it can be observed in **Figure 6A**) is employed. One of the inlets is pierced allowing the introduction of the leaves in the extraction vessel while the STE holder is introduced through another inlet. The effect of stress on the plant can be easily studied with this device. For skin analysis, an extraction vessel that houses the STE holder, as it is depicted in **Figure 6B**, is employed. This approach isolates the skin from the environment thanks to its bell shape allowing the determination of natural volatiles produced by the skin or synthetic volatiles generated after the application of cosmetic products such as perfumes.

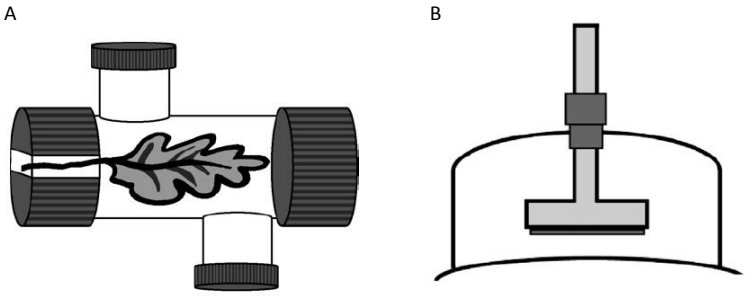


**Figure 6.** Tools for in-vivo sampling: (A) HS-STE of solid samples and (B) DC-STE of biological matrices. Reproduced with permission of Elsevier from Reference [12].

Bichi *et al.* also employed STE to determine potential allergens in cosmetic creams applied in the skin of voluntaries [13]. This approach can be employed to understand the skin absorption of these compounds and also their decay after application. For this purpose, cosmetic creams spiked with the target allergens were applied on the back of the hand of the voluntaries and six PDMS tapes were applied covering a surface of 32 $cm^2$. After the extraction, the tapes were thermally desorbed, the analytes being analyzed by GC. The results confirmed again the potential of STE in this type of studies. Special attention should be given to the recovery obtained since almost all of them fulfilled the 70-130 % criterion.

STE has been adapted to the analysis of lipids composition in cosmetic lipsticks [14]. The cosmetics are applied to a polymeric substrate which is put in close contact with a silica TLC plate that acts a STE material. The compounds in the STE are finally analyzed laser desorption/ionization mass spectrometry imaging.

TFME was employed by Jiang *et al.* for the monitoring of volatile compounds released from human skin, which can be employed for metabolic studies [15]. The extraction can be performed either in DI or HS modes, although the latter approach provides a higher selectivity since it allows the extraction of only compounds with high and medium volatility. In this approach, a circular PDMS film was employed as extractant phase and it is

sandwiched between two stainless steel meshes to avoid its direct contact with the skin surface. The bottom mesh is used as separation between the membrane and the skin whereas the top mesh is employed to fix the whole device into the skin. Finally, the system is covered with aluminum foil to avoid external contamination from the environment. The proposed configuration is evaluated in both in-vial and in-vivo manifolds showing acceptable reproducibility even when different films are employed. In this communication, several interesting variables are evaluated, the appropriate storage conditions as well as the comparison between direct contact and headspace are remarkable. The approach was finally applied to two different metabolic studies. In the first one, some markers of the consumption of garlic are analyzed in the skin emission and on the other case ethanol is determined.

Jahnke *et al.* evaluated the possibilities and limitations of using TFME for the extraction of several polychlorinated biphenyls (PCBs) directly from the fish tissue [16]. For this purpose, disks of PDMS of variable thickness are inserted through slots, previously done with a scalpel, in the fish tissue. For the extraction, the fish is wrapped into aluminum foil and stored during the extraction process in the fridge. After the extraction, the thin films are chemically eluted and the final extract is analyzed by GC/MS. As the lipid composition is not homogeneous in all the fish tissues, several films are placed in different locations and finally pooled before analysis. This technique presents a great potential but the large times required for extraction is the main shortcoming.

## 3. 96-BLADE BRUSHES (THIN FILM) SPME

The increasing research interest to improve current sample preparation technique is directly towards the development of fast and high-throughput analysis [17-21]. Sample extraction rate is enhanced when multiple samples are processed simultaneously. The performance of SPME in a 96-well-plate enables parallel analysis of 96 samples [22]. The first coupling of

TFME to this automatic 96-well-plate was developed by the research group of Prof. Pawliszyn [23]. This system uses 96 small solid phase extraction (SPE) disk membranes fixed to the stainless steel wires for the determination of benzodiazepines in biological samples. With the aim to enhance both the stability and the precision in the development of the coating in the concept of 96-thin film SPME, the same authors developed a flat stainless steel thin film coated with a layer of octadecyl ($C_{18}$) [24]. An automated in-house 96-fiber SPME system for high-throughput was developed. By increasing the contact surface area between the SPME coating and the sample, the recovery of the extraction is noticeably increased. However, in-house multi-fiber device presents some shortcomings such as the instability of the fiber during the agitation or the complications in making the thin film flatten. To overcome these limitations, in 2011 was reported sprayed $C_{18}$-polyacrylonitrile (PAN) coating making use of commercially available automated 96-blade (thin film) technology [25] designed by Professional Analytical System (PAS) (Magdala, Germany). Briefly, the concept 96-blade SPME device is made of a 96-blade SPME device that fits in the 96-well-plate and works as a part of an autosampler. The high throughput analysis of the samples enhances the work rate and simplifies the tedious sample preparation steps. Furthermore, automation and precision process control is achieved since each step is timing regulate. **Figure 7** illustrates a photograph of the current configuration of Concept 96-autosampler [26]. The standard instrument consists of four working positions (each equipped with an orbital shaker) allowing the performance of the following steps: (i) preconditioning, (ii) extraction, (iii) washing and (iv) solvent desorption and (optional) evaporation step.

Prior to the extraction, 96-blade (thin film) SPME is preconditioned to activate in an efficient way the coatings. Typically, a mixture of methanol: water (1:1, v/v) and 15-30 min are employed. As a general rule, taking into account that high stirring speed are generally applied to perform the extraction, the sample volume should avoid the liquid to spill over and possible contaminations of other samples. On the other hand, the selected volume should be large enough to ensure that the totality of the coating is dipped in the sample.

After the extraction and before the desorption, a fast wash step in a weak solvent is needed. This step is mandatory when dealing with biological samples so as to wash properly the coating and avoid the transference of biomolecules to the elution solvent. Usually, a wash with nanopure water for 10-20 second is enough.

Concerning the chemical elution step, two strategies can be employed: (i) direct solvent desorption and (ii) solvent desorption followed by evaporation/reconstitution. Direct solvent desorption should be performed in a large volume to guarantee that the entire length of the extraction phase is eluted. Typically, acetonitrile:water (1:1, v/v) is employed with average desorption time in the range of 40-120 min. The second approach is based on the direct solvent desorption followed by solvent evaporation to dryness and reconstitution into an small volume of solvent suitable for LC. The instrument has an special nitrogen unit so as to carry out both the evaporation of the solvent and analyte enrichment. This strategy is suitable for non-volatile compounds hence analyte loss during evaporation is not an issue. However, it can be also employed with semi volatile analytes if an internal standard is added to quantify analyte losses. A second desorption step in fresh solvent is usually performed in order to avoid undesirable carry-over effects.

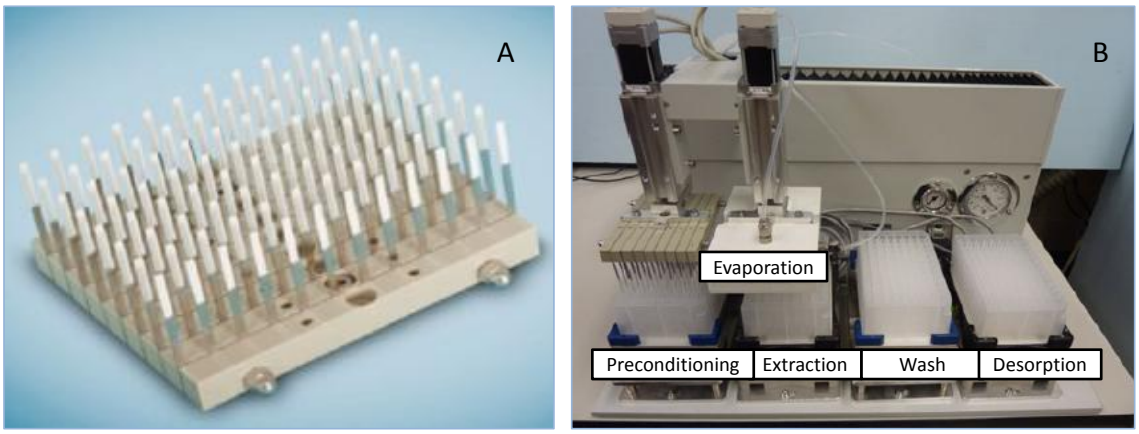


**Figure 7**. (A) 96-blade (thin film) SPME (B) Concept 96-autosampler system. (A) Reproduced with permission of ACS Publications from Reference [25] and (B) adapted from Reference [26] with permission of Springer.

Trends in 96-blade (thin film) SPME systems coating has been in a continuous evolution in the last decade. A biocompatible coating PAN-over $C_{18}$-PAN was applied to the direct analysis of diazepam from whole blood [27]. Polystyrene-divinylbenzene (PS-DVB) PAN and phenylboronic acid (PBA) PAN were employed as coating allowing the simultaneous analysis of different compounds with a wide range of polarity (log P=-3.7 to 2.8) from biological samples [28]. Also $C_{18}$-PAN has been reported to provide high recovery with compounds in wide range of polarity (log P=0.14 to 4.98) [29]. $C_{18}$-strong cation exchange (SCX) is another coating chemistry for the TFME system. This coating has been employed for the determination of carbamazepine and triclosan in wastewater [30]. Later, ionic liquid (IL)-based coatings have been also reported. The synthesis of octadecylimidazolium-modified silica ($SiImC_{18}$) PAN 96-blade SPME was employed as an extraction coating for aminoacids (highly polar nature) from complex matrices coupled to LC-MS/MS analysis [31]. Thin film $C_{18}$-silica glass coating by sol-gel was employed for the determination of benzodiazepines [32]. 96-blade SPME based on the use of carbon nanomaterials was reported for the first time in 2013 [33]. In this proposal, carboxilated multiwalled carbon nanotubes/polydimethylsiloxane (MWCNTs-COOH/PDMS) combines the high adsorption capacity of MWCNTs with the high chemical stability of PDMS. The developed coating is reusable up to 110 extractions and it has been employed for the analysis of phenolic compounds in waters samples. Later, the extraction of these phenolic compounds in complex matrices (wine, berry and grape) has been accomplished making use of PS-DVB-PAN SPME coating [34]. Hydrophilic lipophilic balance (HLB) coated-blades were suitable for the simultaneous determination of nine benzylic and aliphatic quaternary ammonium compounds coupled to LC-MS/MS analysis [35].

## 4. OTHER MICROEXTRACTION TECHNIQUES BASED ON THIN FILMS OR MEMBRANES AS EXTRACTION PHASES

As it was previously indicated, the stirring of the sample during the extraction increases the analyte transference to the extractant phase accelerating the process. Integrated extraction/stirring devices offers additional advantages. On the one hand, the agitation is more intense near to the boundary layer. On the other hand, the potential retention of excessively non-polar compounds on the polymeric stirring bar used is avoided. The use of membrane and thin films as extraction phases in stirring/extraction integrated techniques was proposed almost simultaneously in 2009 by the research groups of Prof. Valcárcel and Prof. Richter. The first research group proposed the stir membrane extraction (SME) [36] technique, which employs a thin polymeric membrane adapted to a stirring device as extraction phase, while the latter proposed the rotating disk sorptive extraction (RDSE) [37] where a thin film of PDMS integrated in a rotating disk is employed to extract the target analytes. The typical extraction units employed in both techniques are presented in **Figure 8.**

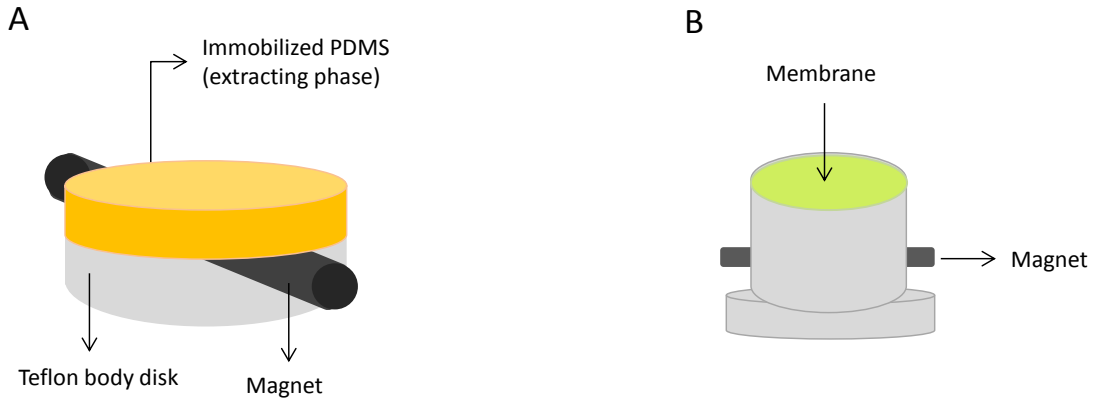


**Figure 8**. (A) Rotating disk sorptive extraction and (B) stir membrane extraction devices.

The typical SME configuration uses polytetrafluorethylene (PTFE) membranes to isolate and preconcentrate hydrophobic analytes. The extraction is performed introducing the extraction unit into the sample and stirring it for a fix time. Later on, the unit is withdrawn from the sample and the membrane can be chemically eluted [36] or directly monitored by a spectroscopic technique [38]. Following both procedures, SME has been applied to the extraction of PAHs and to determine the global hydrocarbon index in water samples, respectively. The comparison of SME with SBSE indicates superior extraction rates that can be ascribed to the planar format of the extractant phase.

RDSE was proposed as an alternative to classic SBSE in order to avoid the contact between the sorptive phase and the vessel walls that may produce the cracking of this phase. This modification let use high-rate stirring in comparison with SBSE, increasing the mass transference from the sample to the sorptive extraction media. The typical extraction procedure involves the introduction of the unit into the sample where it is stirred during a defined time in order to isolate the target compounds. Once the extraction is performed, the unit is taken out from the sample and the PDMS film is eluted [39, 40] or spectroscopically analyzed [41].

## 5. NEW MATERIALS FOR THIN FILM CONSTRUCTION

Although PDMS is the classical phase in TFME, several attempts have been focused on the development of novel sorptive phases. Bagheri and Aghakhani proposed in 2012 the use of polymeric composite membranes of polyaniline (PANI) and Nylon-6 (N6) as sorptive phase under the HS mode for the extraction of several chlorobenzenes from water samples [42]. The PANI-N6 membrane was synthesized by electrospinning over a collector made of aluminum foil providing a polymeric mat with nanofibers diameters in the range of 100–300 nm. For the extraction, the membrane is located in gauze metallic holder which is attached to the upper part of the vial, where the sample is located, to avoid the direct immersion of the membrane. The composite membrane, where the aromatic rings

presented in the PANI structure are the responsible for the extraction of the analytes by π-π interactions, provided an excellent extraction performance allowing the determination of the analytes in the ng/L using GC/MS as instrumental technique.

Saraji *et al.* proposed in 2013 the use of cellulose membranes in TFME [43]. As cellulose is a hydrophilic polymer which cannot interact strongly enough with organic compounds in an aqueous environment, the authors proposed its derivatization to include functional groups able to interact with the target analytes, three estrogenic compounds. Phenyl isocyanate (PHIC), cyclohexyl isocyanate (CHIC), diphenyldichlorosilane (DPDS), and octadecyltrichlorosilane (ODTS) were selected as modifiers of the cellulose membrane and the extraction performance of obtained materials were evaluated. The hydrophobic/hydrophilic balance of the materials was crucial on their extraction performance. On the one hand, the membrane should present a high interaction, usually by non polar and polar forces, with the targets. On the other hand, the membrane should be hydrophilic enough to allow the penetration of the aqueous sample. Considering this, PHIC-cellulose membrane provided the best extraction recoveries.

Kabir *et al.*, who have reviewed the potential of sol-gel phases in sorptive microextraction [44], have recently proposed a novel microextraction technique called fabric phase sorptive extraction (FPSE) [45]. This technique uses a high porosity cellulose membrane as support of a sol-gel organic coating which is uniformly distributed on the network. The high porosity makes easier the penetration of the aqueous phase enhancing the partitioning of the analytes. In addition, the high density of the sol-gel coating, which is 400 times higher than in a conventional SPME fiber, provides FP with a high sorptive capacity. In the first application, FPSE has been applied for the determination of 17α-ethynylestradiol, β-estradiol and bisphenol A (BPA) in urine and water samples by HPLC-FD [46].

## 6. CONCLUSIONS AND TRENDS

TFME has been proved to be an excellent alternative in sample preparation. TFME delivers remarkable advantages such as: (i) high degree of clean-up because of the selectivity towards the target analytes over the matrix interferents (ii) high sensibility since it is a flat film with a high surface area-to-volume ratio of extracting phase and in consequence (iii) short analysis time.

Current research of this technique is focused on two lines of investigation. On the one hand, efforts are mainly toward the development of new coating chemistries. In this context, the reproducibility of the reported coatings can be further enhanced with the development of automatic preparation coating procedures. Furthermore, the synthesis of biocompatible coating with high chemical stability would provide long-term reusability since to date, most of thin-film for bioanalytical purpose are disposable. On the other hand, the development of new thin film configurations would its implementation in environmental, toxicology, forensic and clinic fields. In addition to these two main objectives, research must also focus on the search of novel development for thermal desorption and direct combination with analytical instrumentation. .

## ACKNOWLEDGMENTS

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CAPÍTULO 4

# Stir fabric phase sorptive extraction for the determination of triazine herbicides in environmental waters by liquid chromatography

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**Journal of Chromatography A**

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# Stir fabric phase sorptive extraction for the determination of triazine herbicides in environmental waters by liquid chromatography

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Stir fabric phase sorptive extraction (SFPSE), which integrates sol-gel hybrid organic-inorganic coated fabric phase sorptive extraction media with a magnetic stirring mechanism, is presented for the first time. Two flexible fabric substrates, cellulose and polyester were used as the host matrix for three different sorbents e.g., sol-gel poly(tetrahydrofuran), sol-gel poly(ethylene glycol), and sol-gel poly(dimethyldiphenylsiloxane). The new microextraction device has been analytically evaluated using triazine herbicides as model compounds. The factors affecting the extraction efficiency of SFPSE have been investigated and the optimal extraction conditions have been determined. Under these optimum conditions, the limits of quantification (LOQs) for sol-gel poly(ethylene glycol) coated SFPSE device in combination with UPLC-DAD for the analysis of the seven triazine herbicides were in the range of 0. 26-1.50 µg/L with precision (relative standard deviation) at 2 µg/L concentration ranging from 1.4-4.8% (intra- day, n=5) and 6.8-11.8% (inter-day, n=3). Enrichment factors were found between 444-1411 (compared to 2000 theoretical maximum). Absolute extraction recoveries were in the range of 22.2-70.5%. The developed method was applied for the determination of selected triazine herbicides from three river water samples. Relative recoveries of the target analytes, in the range from 75 to 126%, were found to be satisfactory. The combination of SFPSE with LC-MS/MS allows the improvement of the method sensitivity to the range from 0.015 µg/L to 0.026 µg/L with precision better than 10.8% expressed as relative standard deviation (RSD).

***Keywords***: *Stir fabric phase sorptive extraction (SFPSE); Triazines; Fabric phase sorptive extraction (FPSE); Microextraction.*

## 1. INTRODUCTION

Exponential growth of human activity and rapid industrialization has been implicated to a broad range of chemical pollution all over the world. The development of analytical methodologies is the key in this context not only to make feasible the detection of environmental problems but also to make easier their remediation. These methodologies should face up two main shortcomings, the low concentration of the target pollutants usually in trace and ultra-trace level and the complexity of the sample matrices. Those limitations can be overcome if a rigorous sample preparation strategy, which enhances both sensitivity and selectivity, is performed.

Although conventional extraction techniques such as solid phase extraction (SPE) and liquid-liquid extraction (LLE) are still being widely used for environmental samples, sample treatment has been the focus of intensive research in the last 20 years being miniaturization, automation and simplification the main evolution trends [1]. As a consequence, a number of sample preparation techniques have emerged including sorbent-based sorptive microextraction techniques e.g., solid-phase microextraction (SPME) [2, 3], stir bar sorptive extraction (SBSE) [4, 5], microextraction by packed sorbents (MEPS) [6] and solvent-based sorptive microextraction techniques e.g., single-drop microextraction (SDME) [7, 8], dispersive liquid-liquid microextraction (DLLME) [9] and hollow-fiber microextraction (HF-LPME) [10, 11].

Due to their exceptional characteristics, sorbent-based microextraction techniques have gained wide popularity in a relatively short period of time. As it is known, the performance of those techniques relies directly on both thermodynamic (e.g: type and volume of the sorptive phase) and kinetic (e.g: sample/sorptive phase contact area) factors [12].

Attempts have been made to increase extraction kinetic by increasing the contact surface area of microextraction devices. In this context thin film microextraction, proposed by Pawliszyn *et al.* in 2003 [13], makes use of a thin membrane of polydimethylsiloxane (PDMS)

as extracting phase that favors the close interaction between the sample and sorptive phase. However, the characteristic non-polar nature of PDMS phase limits its applicability to the extraction of hydrophobic analytes and therefore the development of new phase would be desirable. Fabric phase sorptive extraction (FPSE), developed by Kabir and Furton [14], utilizes sol-gel coating technology developed by Malik and co-workers [15] to create an inherently porous hybrid inorganic-organic sorbent material chemically bonded to the flexible and permeable substrate matrix. Due to the strong chemical bonding between the three dimensional network of hybrid inorganic-organic sorbent material, the sol-gel coated extraction medium can be exposed to any organic solvent or solvent mixture of choice. Fabric phases (FPs) are versatile, since they cover a wide range of polarity, and present a high sorptive phase loading which clearly increase the sorption capacity. FPs have been successfully applied to the extraction of selected estrogens form urine samples [16]. The extraction efficiency can also be enhanced if the sorptive phase is integrated in a stirring device following the SBSE model. The integration of planar sorptive phases in such devices has led to the development of novel techniques like rotating disk sorptive extraction (RDSE) [17] and stir-membrane extraction (SME) [18].

In this article, the potential combination of fabric phase sorptive extraction media with the advantages of SME format is presented. The novel approach has been optimized and analytically characterized for the determination of triazines in water by UPLC/DAD. This analytical problem is selected due to the toxicity and persistency of those analytes in the environment. These pollutants should be intensely monitored in natural waters and, taking into account of the restrictive maximum allowable levels established by both the United States Environmental Protection Agency of the (US EPA) and the European Union (EU) [19], a fast, sensitive and efficient sample preconcentration step is warranted.

## 2. EXPERIMENTAL

### 2.1. Instruments

An Eppendorf Centrifuge Model 5415 R (Eppendorf North America Inc. USA) was used to centrifuge different solutions to obtain particle free sol solutions for the sol-gel coating. A Fisher Scientific Digital Vortex Mixer (Fisher Scientific, USA) was employed for thorough mixing different solutions. A 2510 BRANSON Ultrasonic Cleaner (Branson Inc., USA) was used to prepare bubble-free sol solution. A Barnstead NANOPure Diamond (Model D11911) deionized water system (Dubuque, IA) was used to obtain ultra-pure deionized water (18.2 MΩ) for sol-gel synthesis. A Perkin Elmer Spectrum 100 FT-IR Spectrometer equipped with Universal ATR Sampling Accessory (Santa Clara, CA) was used to perform FT-IR characterization of fabric substrates and sol-gel coated FPSE media. A Philips XL 30 Scanning Electron Microscope equipped with an EDAX detector was employed to obtain SEM images of coated and uncoated FPSE media.

### 2.2. Materials, reagents and samples

The reagents used were of analytical grade or better. Sol-gel active polymers poly(tetrahydrofuran), poly(dimethyldiphenylsiloxane), and poly(ethylene glycol) were purchased from Sigma-Aldrich (St. Louis, Mo, USA), Gelest (Morrisville, PA, USA), AlfaAesar (Ward Hill, MA, USA), respectively. Acetone, dichloromethane, methyltrimethoxysilane (MTMS), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Sodium hydroxide and hydrochloric acid were purchased from Thermo Fisher Scientific (Milwaukee, WI, USA). Substrates for fabric phase sorptive extraction media were obtained from Jo-Ann Fabric (Miami, FL, USA). For sol-gel PTHF and sol-gel PEG coating, 100% cotton cellulose fabric and for sol-gel poly(dimethyldiphenyl siloxane) coating, 100% polyester fabric were used as the substrate. The seven triazine herbicides simazine (SMZ), atrazine (ATZ), secbumeton (SBM),terbumeton (TBM), propazine (PPZ), prometryn (PMT) and terbutryn (TBT) were purchased from Sigma–Aldrich (Madrid, Spain). Stock standard

solutions of each analyte were prepared in acetonitrile (Sigma–Aldrich) at a concentration of 1 g/L, except of simazine which was prepared a 500 mg/L. Working solutions were prepared on a daily basis by rigorous dilution of the stocks in Milli-Q water (Millipore Corp., Madrid, Spain) or acetonitrile as required. Sodium chloride from Sigma-Aldrich (St. Louis, Mo, USA) was used to adjust the ionic strength of the standards and samples.

Different environmental water samples from Córdoba, Spain, were analyzed. All the samples were collected in amber-glass bottles without headspace and stored in the dark at 4 ºC until their analysis. Prior to the stir fabric phase extraction, no filtration of the environmental water samples was carried out.

## 2.2. Preparation of sol-gel poly(dimethyldiphenysiloxane) (PDMDPS), poly(tetrahydrofuran) (PTHF) and poly(ethylene glycol) (PEG) coated fabric phase sorptive extraction media

### *2.2.1. Pretreatment of fabrics for sol-gel coating*

100 $cm^2$ segments of cellulose and polyester fabrics were first soaked with deionized water for 15 min under constant sonication. The fabrics were then cleaned with copious amount of deionized water, followed by treating with 1 M NaOH solution for 1 h under sonication. The mercerized fabrics were then washed several times with deionized water, followed by treating with 0.1 M HCl solution for 1 h under sonication. The treated fabrics were then washed with copious amount of deionized water and finally dried overnight in an inert atmosphere. The dried fabrics were stored in clean airtight glass containers until they are coated with sorbents.

### *2.2.2. Preparation of the sol solutions for coating the substrate*

The sol solution for creating the sol-gel PTHF coating was prepared by using a modified version of a previously described formulation [20]. Briefly, the sol solution was prepared by dissolving 10 g of poly-THF 250 polymer, 10 mL methyltrimethoxysilane sol-gel precursor

(MTMS), 20 mL methylene chloride/acetone (50:50 v/v) as the organic solvent system, 4 mL trifluoroacetic acid (5% water) as the sol-gel catalyst. The mixture was then vortexed for 3 min, centrifuged for 5 min and finally the clear supernatant portion of the sol solution was transferred to a clean 60 mL amber colored glass bottle. The sol solution for creating sol-gel PDMDPS and sol-gel PEG coatings were prepared in the same way but instead of PTHF, 10 g of PDMDPS or PEG was added, respectively.

### *2.2.3. Formation of sol-gel PTHF, sol-gel PDMDPS, and sol-gel PEG coatings on the substrate*

Polyester fabric was used as the substrate for sol-gel PDMDPS, whereas cellulose fabric was used as the substrate for sol-gel PTHF and sol-gel PEG coatings. The clean and treated fabrics were gently inserted into the reaction bottle containing the sol solution so that a three-dimensional network of sol/gel PDMDPS, PTHF or PEG can be formed throughout the porous substrate matrix. The fabrics were kept inside the sol solution for a pre-determined period of time (2 h for PTHF/sol-gel and PTHF/sol-gel, whereas the optimum residence time for PEG/sol-gel was found to be 1 h). Upon completion of the coating period, the sol solution was expelled from the reaction bottle and the coated fabric was dried and aged in a home-made conditioning device built inside a gas chromatography oven with continuous helium gas flow at 50 °C for 24 h. Before using for stir fabric phase sorptive extraction, the sol-gel PDMDPS/sol-gel PTHF/sol-gel PEG coated fabric were rinsed sequentially with methylene chloride and methanol followed by drying at 50 °C under an inert atmosphere for 1 h. The fabric phase sorptive extraction media coated with sol-gel PDMDPS/ sol-gel PTHF/sol-gel PEG were then cut into appropriate size suitable for the SFPSE device and stored in a closed glass container to prevent from any contamination.

### 2.3. Chromatographic analysis

Two chromatographic systems, including different detectors, were employed in the development of the present research. The optimization of the extraction procedure and its validation was carried out on a Waters-AcquityTM Ultra Performance LC system (Waters Corp., Madrid, Spain) using an Acquity UPLC® BEH $C_{18}$ column (1.7 μm particle size, 2.1 mm × 50 mm) maintained at 43 ºC. The mobile phase consisted of (A) water and (B) acetonitrile at a flow rate of 0.5 mL/min using a gradient elution program. The initial composition was fixed at 75% A, the percentage being decreased to 56.6% in 5 min. The injection volume was 5 μL with partial loop with needle overfill mode. The separated analytes were determined using a PDA eλ (extended wavelength) Detector (Waters) at 223 nm. System control was achieved with Empower software.

The extraction performance is calculated in relative terms (extraction recovery and enrichment factors) and the obtained values are independent of the instrument employed. For this reason, LC-MS/MS was finally evaluated as instrumental technique since it is the reference instrumental technique in environmental analysis. Chromatographic analysis was performed on an Agilent series 1260 HPLC system equipped with a pump, a degasser, an autosampler and a thermostated column compartment (AgilentTechnologies, Palo Alto, CA, USA). Chromatographic separation was carried out at 40 ºC on an Agilent Poroshell 120 SB-$C_{18}$ analytical column (2.1 mm × 75 mm, 2.7 μm). The mobile phase comprised 5 mM acetate ammonia aqueous solution (solvent A) and LC-MS grade methanol (solvent B) at a flow rate of 0.2 mL/min and it was operated under the gradient elution mode. The gradient program was as follows: 0.0-8.0 min, 55% B; 8.0-11.0 min, 55-70% B; and back to 55% B in 1 min. Equilibration time was fixed at 8 min. 5 μL was selected as injection volume.

MS spectra were conducted on an Agilent 6420 triple quadrupole mass spectrometer equipped with electrospray ionization source (Agilent Corporation, MA, USA). Quantification was performed in positive using multiple reaction monitoring (MRM). Instrument parameters were as follows: source temperature, 300 ºC; cone gas flow, 10

L/min; capillary voltage, 2000V, nebulizer gas flow, 60 psi and cell accelerator voltage 7V. Nitrogen (99.995%) was used as cone, desolvation and collision gas. Dwell time was set at 200 ms. Optimized parameters for each compound are also listed together with the mass transitions in **Table 1**. Data acquisition was performed with MassHunter Workstation (Agilent Technologies, USA).

**Table 1.** Transitions, retention time and optimized potentials for LC–MS/MS analysis.

| Compound | Retention time (min) | Transitions | Fragmentor (V) | CE (eV) |
|---|---|---|---|---|
| Simazine | 2.37 | 201.9 → 131.9[a] | 114 | 13 |
| | | 201.9 → 104[b] | 114 | 24 |
| Atrazine | 3.47 | 215.9 → 173.9[a] | 117 | 14 |
| | | 215.9 → 96[b] | 117 | 23 |
| Secbumeton | 5.04 | 226.2 → 169.9[a] | 125 | 14 |
| | | 226.2 → 100[b] | 125 | 24 |
| Terbumeton | 5.50 | 226 → 169.9[a] | 104 | 14 |
| | | 226 →114[b] | 104 | 25 |
| Propazine | 6.27 | 229.9 → 145.9[a] | 129 | 14 |
| | | 229.9 → 187.9[b] | 129 | 20 |
| Prometryn | 9.17 | 242 → 157.9[a] | 140 | 21 |
| | | 242 → 199.9[b] | 140 | 14 |
| Terbutryn | 10.36 | 241.9 → 185.9[a] | 108 | 13 |
| | | 241.9 → 71.1[b] | 108 | 26 |

[a]MRM transition used for quantification; [b] MRM transition used for confirmation; CE, Collision energy.

## 2.4. Stir fabric phase sorptive extraction (SFPSE) unit

The SFPSE unit, as it is described elsewhere [15], is constructed using four elements: (a) a section of a 3-ml polypropylene SPE cartridge; (b) an iron wire; (c) fabric phase sorptive extraction media and (d) a external element which is cut from a 5 mL pipette tip.

The different building elements as well as the assembly process are depicted in **Figure 1.** The fabric phase sorptive extraction media was placed in the upper part of the internal cylinder, and the unit was sealed by displacing the external element through the internal one. The fabric phase sorptive extraction medium coated with appropriate sol-gel sorbent coating was tightly fixed to prevent any displacement during extractions. Once sealed, the extraction device was pierced with an iron wire (1.4 cm long) to allow the magnetic stirring of the unit.

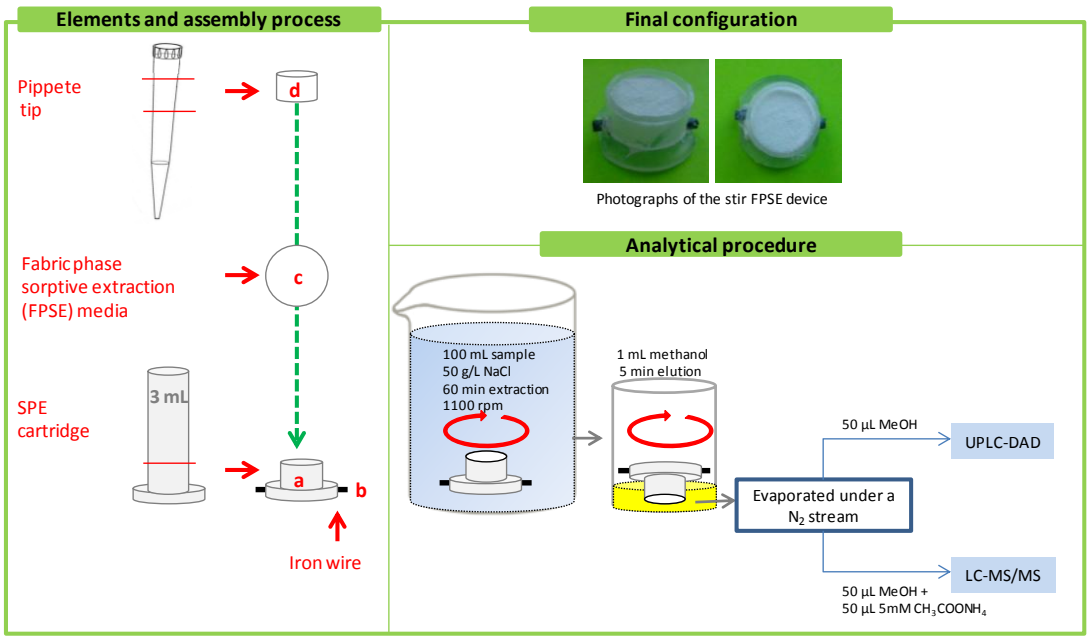


**Figure 1.** Stir fabric phase sorptive extraction device: components and assembly process.

### 2.5. Analytical procedure

The extraction procedure was as follows: 100 mL of the aqueous standard or environmental sample were added to a beaker which is subsequently placed in a magnetic stirrer. The ionic strength of the solution was adjusted adding sodium chloride at a final concentration of 50 g/L. In a second step, the stir fabric phase sorptive extraction unit, previously conditioned with methanol, was introduced in the beaker and stirred at 1100 rpm for 60 min; the extraction of the analytes was performed. Once finished, the extraction unit was withdrawn from the solution by means of tweezers. The unit was immersed faced down in 1 mL of methanol (only the fabric phase being in close contact with the solvent) and kept under magnetic stirring for 5 min for analyte elution. To improve the sensitivity and precision of the measurements, the extract was evaporated to dryness under a nitrogen stream and the residue is finally re-dissolved in a lower volume. For UPLC-DAD analysis the final residue was re-dissolved in 50 μL of methanol while for LC-MS/MS analysis, the residue was re-dissolved in 50 μL of methanol which was subsequently mixed with 50 μL of a 5mM ammonium acetate aqueous solution to improve the chromatographic separation.

Between extractions, the stir fabric phase sorptive extraction unit was magnetically stirred in 2 mL of methanol. No carry-over effects were observed following this cleaning protocol.

## 3. RESULTS AND DISCUSSION

### 3.1. Chemistry of the SFPSE substrates and the sol-gel coatings

A large number of commercially available natural and synthetic fabrics were considered as the potential candidates for the substrate of fabric phase sorptive extraction including cellulose, polyester, nylon, and polyamide. All these fabrics either contain readily available sol-gel active functional groups or may have the capability to contain sol-gel active functional groups *via* surface modification. However, polyester and cellulose fabrics

inherently possess sol-gel active functional groups and therefore both the fabrics were selected as the substrates for sol-gel coating. Cellulose fabric is known to be hydrophilic and polyester fabric is hydrophobic [21]. Since both the substrate and the sol-gel coating contribute to the final selectivity and polarity of the resulting extraction media, a relatively nonpolar organic polymer poly(dimethyldiphenylsiloxane) was used as the organic polymer to coat hydrophobic polyester substrate in order to create a nonpolar extraction media. Poly(tetrahydrofuran) and poly(ethylene glycol) polymers are medium polarity and high polarity organic polymers, respectively. As such, they were used to coat hydrophilic cellulose substrate in order to develop extraction media with medium and high polarities.

The chemical reactions involving the sol-gel coating process is well studied and widely reported [22]. The creation of sol-gel hybrid organic-inorganic coating on the substrate involve: (1) controlled catalytic hydrolysis of the sol-gel precursor, MTMS; (2) polycondensation of hydrolyzed MTMS, leading to a growing three-dimensional sol-gel network; (3) random incorporation of sol-gel active polymers into the evolving sol-gel network; (4) chemical immobilization of the growing sol-gel network *via* condensation to the flexible cellulose and polyester substrates. In this final polycondensation, two properly aligned hydroxyl moieties are fused together to elongate the chain, accompanied by the elimination of low-molecular weight by product, e.g. water. A general reaction scheme for creating sol-gel hybrid organic-inorganic coating on the substrate is shown in **Figure 2.**

During the polycondensation, the growing sol-gel network reacts with available surface hydroxyl groups of cellulose or terminal hydroxyl groups of polyester microfibrils (submicroscopic elongated bundles that form individual fiber strand) resulting in a covalently bonded sol-gel hybrid coating uniformly distributed throughout the substrate matrix with characteristic high solvent and chemical stability as well as highly accessible active sites for efficient and fast analyte extraction. A schematic of sol-gel poly(ethylene glycol) coated FPSE media is shown below in **Figure 3**.

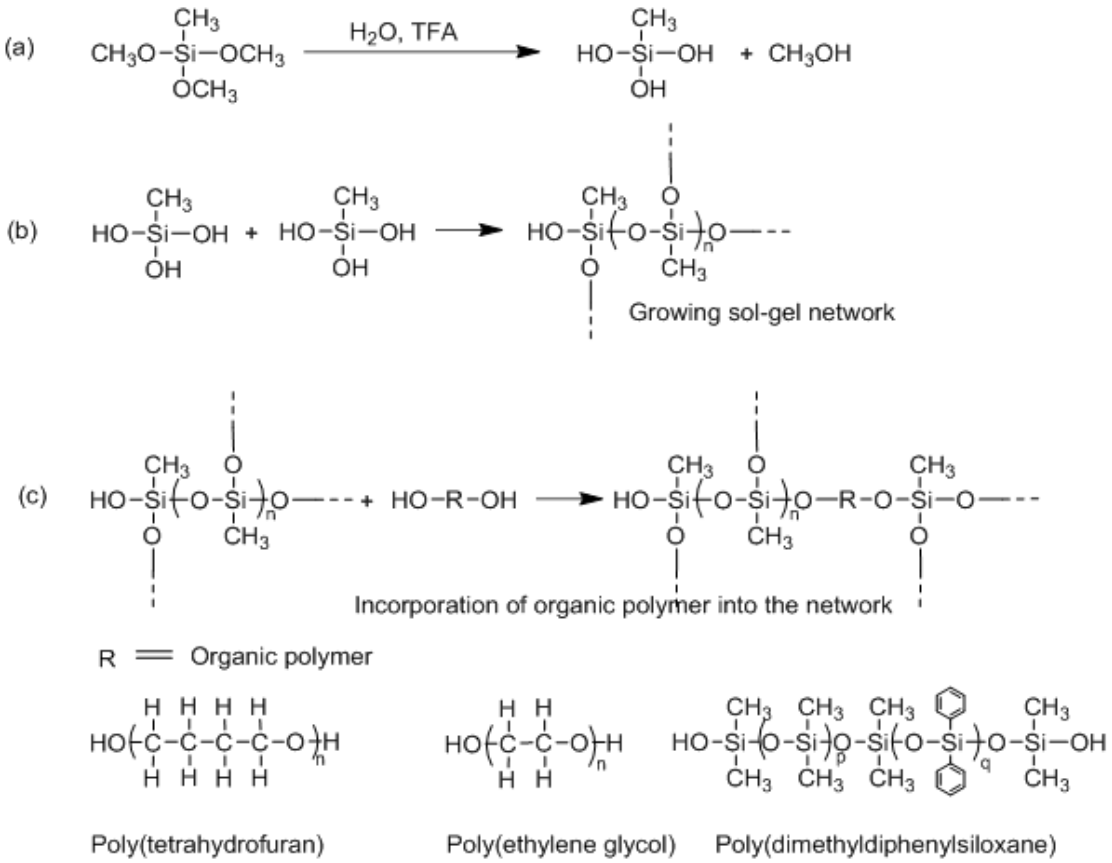


**Figure 2.** Chemical reactions involved in the synthesis of sol-gel hybrid organic-inorganic sorbents.

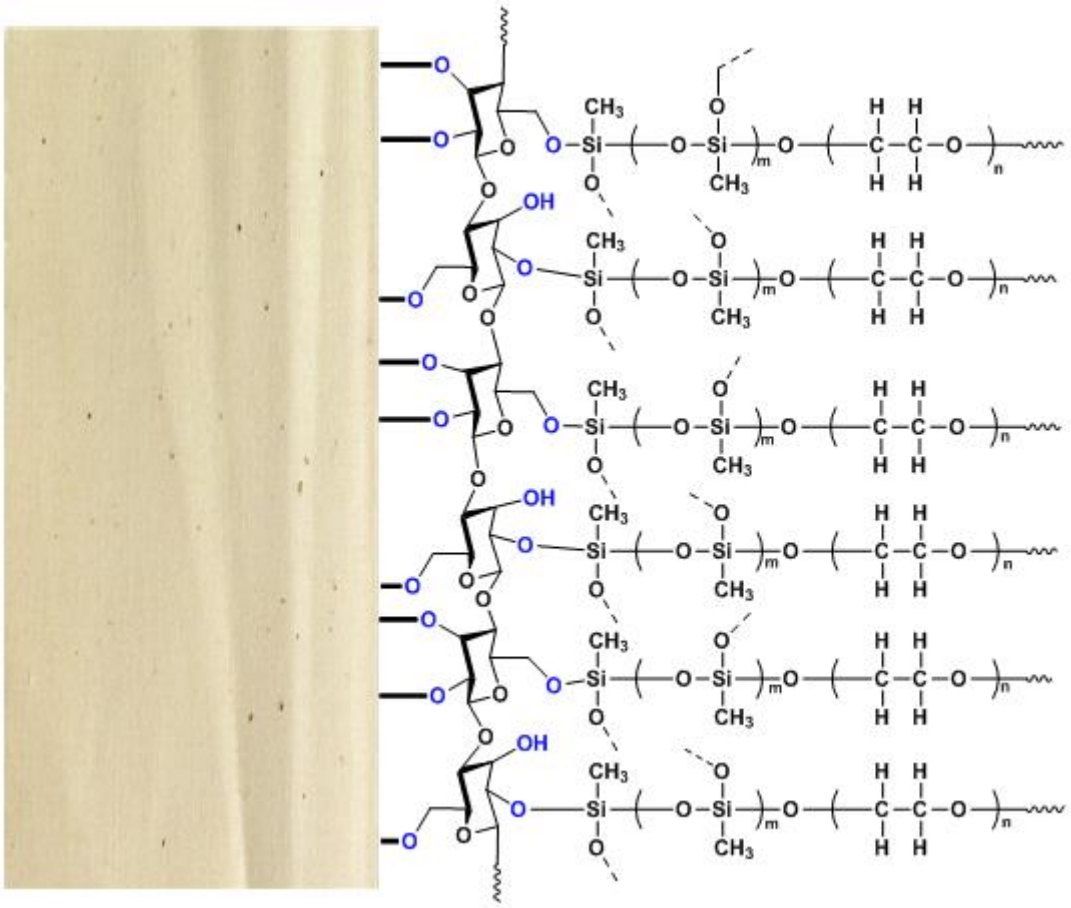

**Figure 3.** Schematic representation of sol-gel poly(ethylene glycol) coated FPSE medium.

### 3.2. Characterization of sol-gel PTHF/PDMDPS/PEG coated FPSE media

The morphology of uncoated FPSE media and the sol-gel poly(ethylene glycol) coated FPSE media were investigated by scanning electron microscopy (SEM). **Figure 4** displays scanning electron micrographs of: (a) uncoated cellulose fabric at 100 x magnification; (b) sol-gel PEG coated FPSE media at 100 x magnifications; (c) uncoated cellulose fiber at 1000

x magnification, and (d) sol-gel PEG coated cellulose fiber at 4000 x magnification. As can be seen from these SEM images, the cellulose fabric has distinct porous structure that is well preserved even after the sol-gel coating. The presence of sol-gel coating is barely visible due to its uniform distribution throughout the three dimensional matrix of the substrate. As such, it can be ascertained from the SEM image of sol-gel poly(ethylene glycol) coated FPSE media that the coating was not only limited to the cellulose substrate surface, rather it was uniformly distributed throughout its three dimensional matrix. The SEM images of sol-gel PTHF and sol-gel PDMDPS were found to have similar morphologies which are not shown here, since these phases were not the optimal phases (see optimization of the extraction conditions) for the target analytes.

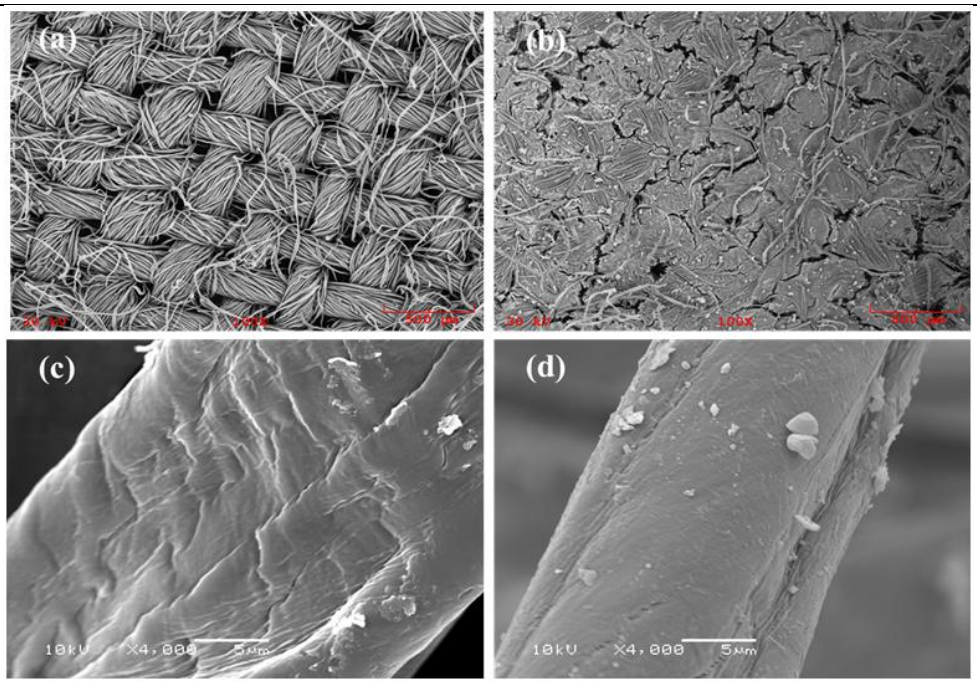


**Figure 4.** Scanning electron microscopy images of (a) uncoated cellulose fabric at 100 x magnification; (b) sol-gel PEG coated FPSE media at 100 x magnifications; (c) uncoated cellulose fiber at 1000 x magnification, and (d) sol-gel PEG coated cellulose fiber at 4000 x magnification.

### 3.3. Optimization of the extraction conditions for triazine herbicides

Different variables, which are summarized in **Table 2**, may affect the efficiency of the extraction procedure and therefore their effects on the analytes extraction were considered in depth in the optimization process. **Table 2** also reflects their initial values, the interval studied and the optimum values for each variable. The optimization was performed under a univariate approach although a couple of variables (sample volume and the stirring rate) were considered jointly since they are directly related. An aqueous standard solution containing the seven triazine herbicides at a concentration of 50 µg/L was used in these studies. Sol-gel PEG coated FPSE medium was employed in this study.

**Table 2.** List of the variables involved in the stir fabric phase sorptive extraction process.

| Variable | Initial value | Interval studied | Optimum value |
|---|---|---|---|
| Fabric phase | | PTHF, PDMDPS, PEG | PEG |
| Ionic strength (g/L NaCl) | 50 | 0 - 200 | 50 |
| Sample volume (mL) | 100 | 25 - 200 | 100 |
| Stirring rate (rpm) | 1100 | 0 - 1100 | 1100 |
| Extraction time (min) | 30 | 5 - 120 | 60 |
| Elution solvent | Methanol | Methanol, acetonitrile, acetone | Methanol |
| Elution modality | Ultrasonic stirring | Ultrasonic stirring, magnetic stirring, percolation | Magnetic stirring |
| Elution volume (mL) | 1 | 0.5 - 2 | 1 |

#### *3.3.1 Selection of the fabric phase*

Three different fabric phase sorptive extraction media coated with sol-gel PEG, sol-gel PTHF and sol-gel PDMDPS were evaluated for the extraction of triazine herbicides from aqueous solutions using the absolute extraction recovery (AER) as evaluating parameter. The results, which are summarized in **Table 3**, indicated that triazines are better extracted by sol-gel

PEG (AERs in the range from 17.8% to 53.3%) followed by sol-gel PTHF (AERs in the 4.4%-22.8% interval) and sol-gel PDMDPS (AERs in the range from 0.6 to 9.4%). Therefore, sol-gel PEG was selected as the sorptive phase for further studies.

**Table 3**. Extraction performance, expressed as absolute extraction recoveries (AERs), of different fabric phase sorptive extraction media towards triazine herbicides.

| Analyte | AERs (%) Fabric Phase Sorptive Extraction Media | | |
|---|---|---|---|
| | **PTHF** | **PDMDPS** | **PEG** |
| Simazine | 4.4 | 0.6 | 17.8 |
| Atrazine | 8.0 | 1.1 | 27.4 |
| Secbumeton | 13.1 | 1.7 | 39.6 |
| Terbumeton | 10.4 | 4.9 | 47.6 |
| Propazine | 9.7 | 6.2 | 40.3 |
| Prometryn | 18.4 | 6.3 | 53.0 |
| Terbutryn | 22.8 | 9.4 | 58.8 |

### *3.3.2. Effect of the ionic strength on the extraction*

Ionic strength may play a key role in microextraction techniques. In fact, it may present two contradictory effects. On the one hand, it can decrease the solubility of the analytes with intermediate polarity in water and thus favor their transference to the extraction media (salting-out effect). On the other hand, ionic strength may increase the viscosity of the sample, negatively affecting the extraction kinetics. This aspect affects to the same degree to all analytes regardless of their polarity.

The influence of the ionic strength was evaluated in the range of 0 to 200 g/L using sodium chloride (NaCl) as model electrolyte. The extraction of all the analytes is positively affected at lower ionic strength (showing a prevalence of the salting out effect) whereas at higher concentration (over 50 g/L) a decrease in the EFs was observed (due to the second effect). The results are shown in *Supplementary Material* **Figure S1.** However the extraction of

simazine and atrazine, the less hydrophobic analytes, present a slightly increase in the EFs with the ionic strength.

Attending to the results, 50 g/L of sodium chloride was selected as optimum value for further studies.

### *3.3.3. Effect of the stirring rate and sample volume on the extraction*

The sample volume and the stirring rate are related variables. Generally, when higher samples volumes are processed, higher stirring rates should be used. For this reason, both variables were optimized together considering four different sample volumes (25, 50, 100 and 200 mL) and five different stirring rates (0, 275, 410, 825 and 1100 rpm). The optimization process was performed taking into account all the target analytes, but for simplicity, **Figure 5** shows the bivariant effect on the extraction (EFs) of prometryn by means of a contour surface graph. The results obtained for the rest of the analytes are presented in the *Supplementary Material* (**Figures S2, S3, S4, S5, S6 and S7**).

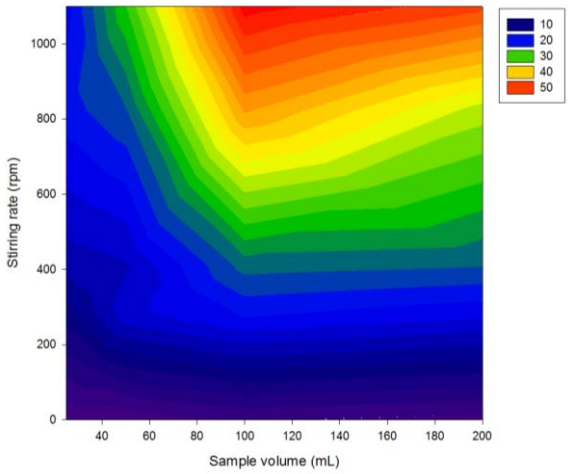


**Figure 5**. Bivariant effect of the sample volume and stirring rate on the analytical signal obtained for a standard solution containing prometryn at 50 µg/L.

Three different behaviors were observed depending on the sample volume. For 25 mL, the EFs increased with the stirring rate up to 825 rpm. For higher velocities, a vortex was created directly above the extraction device reducing the contact between the sample and the fabric phase sorptive extraction media. When 50 mL of sample volume was employed, the EFs for the most hydrophilic analytes increases with the stirring rate up to 825 rpm remaining constant for higher velocities, whereas the EFs for the most hydrophobic analytes increased linearly. When 100 and 200 mL of sample volume was employed, the EFs increased almost linearly with the stirring rate. No significant differences were observed in terms of the EFs for 100 and 200 mL, which can be ascribed to the limited capacity of the unit to homogenize larger samples volumes. According to the obtained results, a sample volume of 100 mL and a stirring rate of 1100 rpm were chosen as the optimal values to carry out the extraction procedure.

### *3.3.4. Effect of the extraction time*

The influence of the extraction time or stirring time is also an important variable in the extraction process. This variable was investigated in the range from 5 to 120 min and the results are depicted in **Figure 6.** From 5 to 60 min, the extraction markedly increases with the extraction time while from 60 to 120 min the increase is less pronounced. As other sorptive techniques, like SPME or SBSE, stir fabric phase sorptive extraction requires a longer time to reach the partition equilibrium. For this reason, the extraction time is selected as a compromise between sensitivity (signal or enrichment factor) and sample throughput (number of samples that can be processed). Finally, 60 min was fixed as the optimum value for further studies.

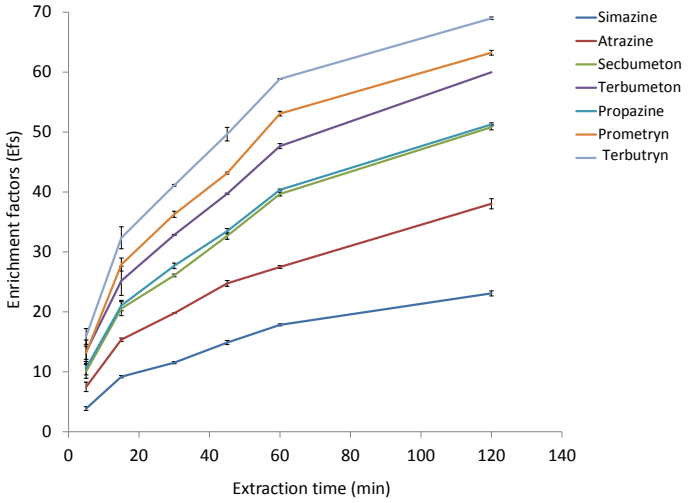


**Figure 6.** Effect of the extraction time on the enrichment factors of the analytes.

### *3.3.5. Elution process*

Once the analytes have been conveniently extracted, they should be eluted for the subsequent chromatographic analysis.

Three different elution solvent were tested, namely: methanol, acetonitrile and acetone, being methanol selected since it provided the best results in terms of sensitivity and precision.

Three different elution strategies using methanol as solvent, namely: ultrasonic stirring, magnetic stirring and elution by percolation, were evaluated. According to the sensitivity and precision (see **Figure S8**) of the different modes, magnetic stirring was selected as the best approach. The elution volume was studied was studied in the range from 0.5 to 2 mL as it can be observed in **Figure S9**. 1 mL of methanol was selected as the optimum value. Finally, the elution time (defined as the contact time methanol fabric phase) was evaluated.

According to the results, shown in **Figure S10**, 5 min were enough to elute the analytes and therefore this value was selected as optimum value.

After optimization, the enrichment factors were in the range from 20 to 60. In order to improve these values, and therefore the sensitivity of the method, the final extract was evaporated under a $N_2$ stream and re-dissolved in a lower volume for chromatographic analysis. This step is possible since no losses by evaporation are observed for the target analytes. For UPLC-DAD analysis the final residue was re-dissolved in 50 μL of methanol allowing the enhancement of the preconcentration factors 20 times. For LC-MS/MS analysis, the residue was re-dissolved in 50 μL of methanol which was subsequently mixed with 50 μL of a 5 mM ammonium acetate solution to improve the chromatographic separation. In the latter case, the enrichment factors were enhanced 10 times.

### 3.4. Analytical figures of merit

The analytical figures of merit of the proposed method are summarized in **Table 4.** The calibration curve for triazine herbicides was constructed by using seven working aqueous standards prepared at controlled concentrations which were subjected to the optimized extraction procedure. The method was characterized on the basis of its linearity, sensitivity, precision and accuracy.

The sensitivity of the method was evaluated according to the limit of detection (LOD) and quantification (LOQ). LOD, calculated using a signal-to-noise ratio (S/N) of 3 ranged from 0.08 μg/L (terbumeton) to 0.47 μg/L (prometryn) while the LOQ, calculated using a S/N of 10, varied between 0.26 μg/L (terbumeton) and 1.50 μg/L (prometryn).

**Table 4.** Analytical figures of merit of the proposed method for the determination of triazine herbicides in water.

| Analyte | UPLC-DAD | | | | EFs[d] | AER[e](%) | LC-MS/MS | |
|---|---|---|---|---|---|---|---|---|
| | LOD[a] (ng/) | LOQ[b] (µg/) | Precision[c] (%) at 2 µg/L | | | | MDL[f] (ng/) | Precision[c] (%) at 100 ng/L (n=5) |
| | | | Repeatability (n=5) | Reproducibility (n=3) | | | | |
| Simazine | 140 | 460 | 4.6 | 7.9 | 444 | 22.2 | 26.8 | 9.5 |
| Atrazine | 240 | 790 | 3.0 | 11.8 | 729 | 36.5 | 19.2 | 7.8 |
| Secbumeton | 80 | 260 | 4.8 | 8.1 | 988 | 49.4 | 21.9 | 9.3 |
| Terbumeton | 80 | 260 | 3.8 | 6.8 | 1165 | 58.2 | 17.9 | 7.4 |
| Propazine | 110 | 360 | 4.5 | 9.0 | 996 | 49.8 | 22.5 | 10.8 |
| Prometryn | 470 | 1500 | 1.4 | 11.7 | 1286 | 64.3 | 17.1 | 7.1 |
| Terbutryn | 80 | 260 | 3.8 | 7.1 | 1411 | 70.5 | 15.6 | 6.9 |

[a] *LOD,* limit of detection, calculated for a S/N of 3.
[b] *LOQ,* limit of quantification, calculated for a S/N of 10.
[c] Precision expressed as relative standard deviation.
[d] EFs, enrichment factors. These values are calculated for UPLC-DAD analysis where the final extract is 50 µL in volume. For LC-MS/MS analysis the values should be divided by two since the final extract volume is 100 µL. The theoretical maximum value for EF is 2000 for UPLC-DAD and 1000 for LC-MS/MS.
[e] *AER,* absolute extraction recovery.
[f] MDL, method detection limit.

The precision (expressed as relative standard deviation, RSD) was evaluated under repeatability (intra-day) and reproducibility (inter-day) conditions at 2 µg/L concentration level. Different extraction devices have been used in this study. As it can be seen in **Table 4,** the repeatability (n=5) ranged from 1.4% to 4.8%. The reproducibility (n=3) ranged from 6.8 to 11.8%. Moreover, **Table 4** also summarized the enrichment factors (EFs) obtained which were in the range from 444 (simazine) to 1410 (terbumetryn). These EFs involve absolute extraction recoveries (AER) from 22.2% to 70.5%.

Once optimized and analytically characterized, the proposed method was applied for the determination of the target triazines in environmental water samples. A recovery study was performed in order to evaluate the applicability of the proposed method to determine triazine herbicides in stream and river waters. First of all, the samples were analyzed in order to find any potential presence of the analytes. Since the analytes were not detected, the samples were spiked at 2 µg/L concentration level. From the results listed in **Table 5**, it can be concluded that the extraction process fulfills the 70-130% recovery criterion [23]. The results show the potential of the proposed stir fabric phase sorptive extraction for extracting seven triazine herbicides from environmental waters.

**Table 5.** Relative recovery study performed on real samples spiked with the analytes at a concentration of 2µg/L.

| Analyte | Environmental water samples (R% ± SD) | | |
|---|---|---|---|
| | **Stream I** | **Stream II** | **River I** |
| Simazine | 124 ± 5 | 84 ± 4 | 108 ± 4 |
| Atrazine | 126 ± 3 | 75 ± 3 | 94 ± 3 |
| Secbumeton | 102 ± 4 | 76 ± 5 | 103 ± 5 |
| Terbumeton | 101 ± 3 | 75 ± 4 | 104 ± 3 |
| Propazine | 96 ± 5 | 75 ± 4 | 97 ± 4 |
| Prometryn | 111 ± 2 | 78 ± 1 | 104 ± 1 |
| Terbutryn | 99 ± 4 | 78 ± 3 | 99 ± 4 |

*R%* extraction recovery, *SD* standard deviation (n=3).

Finally, the proposed extraction technique was combined with LC-MS/MS as it is the reference technique in environmental analysis. The extraction procedure of the sample was almost the same than that applied for UPLC-DAD analysis, the final elution step being performed in a slightly different way. As it was expected, the sensitivity of the methodology was improved as it can be shown in **Table 4**. In this case, the method detection limits (MDL)

for all the analytes were in the range from 15.6 ng/L to 26.8 ng/L. The precision of the determination was calculated at 100 ng/L and the RSD ranged from 6.8 to 10.8%.

Finally, **Table 6** compares the proposed method with other methodologies used to solve the same analytical problem [24-41]. These approaches cover a wide range of extractions modes as well as instrumental techniques. For simplicity, the comparison has been focused on those techniques that work under solid phase (micro) extraction principles considering some factors like extraction time, sample volume, LOD, relative recovery and RSD. Relatively long extraction time was required in almost all the applications. According to the results, the UPLC-DAD provides precision levels comparable with the other approaches. The sensitivity of the UPLC-DAD can be considered the weak factor although this fact can be explained if the instrumental technique is considered in the discussion. On the one hand, the use of MS provides the best sensitivity levels in most cases [24-26, 31, 39]. However, the UPLC-DAD method provides similar or better results than those given with UV or DAD detection [27, 29, 32, 41] and even comparable detection limits with some MS applications [33, 36]. The use of LC-MS/MS instead of DAD as instrumental technique clearly improves the sensitivity, in the range from 5 to 18 times depending on the analyte, even when the EFs are lower due to the different final extracts volume.

## 4. CONCLUSIONS

A novel sorption-based microextraction procedure with integrated stirring mechanism, SFPSE was proposed. The new approach integrates the advantages of sol-gel hybrid organic-inorganic sorbents, rich surface chemistry of natural and synthetic fabrics, and fast analyte diffusion mediated by built-in stirring mechanism. Due to high sorbent loading, extended contact surface area provided by the device design, and fast analyte diffusion, SFPSE have shown high analyte enrichment capacity. The combination of SFPSE with UPLC-DAD for the analysis of triazine herbicides from aqueous solutions have provided LODs at

low µg/L with excellent within day (RSD% 1-4-4.8) and between days reproducibility (RSD% 6.8-11.8). The combination of SFPSE with LC-MS/MS allows the improvement of the detection limit to the low ng/L range. Absolute recovery of individual analyte was in the range of 22.2-70.5%, which was unprecedented in any microextraction devices. The simplicity in design, easy to use, portability, and wide range of readily available sol-gel based sorbents with tunable selectivity and porosity have made SFPSE a highly promising technique for environmental, pharmaceutical, toxicological, clinical, and forensic applications.

**Table 6**. Comparison of the proposed extraction procedure with other published methods to solve the same analytical problem.

| Extraction technique[a] | Extractant[b] | Sample volume (mL) | Instrumental technique[c] | Extraction time (min) | LODs[d] (µg/L) | EFs[e] | Relative recovery (%) | Precision (% RSD[f]) | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| SPE | MWCNTs on disk filter | 200 | GC-MS | | 0.0025–0.005 | 3900-4000 | 87-110 | 3.0-6.9 | [24] |
| SPE | $C_{18}$ cartridge | 300-1000 | GC-MS | >30 | 0.005-0.00167 | -- | 90.5 | 3.5 | [25] |
| SPME | CW/DVB | 3 | GC-MS | 30 | 0.02-0.06 | -- | | <10 | [26] |
| SPME | Fiber CW/TPR<br>Fiber PDMS/DVB | 3 | HPLC-UV | 20 | 2.8-3.4<br>1.2-2.6 | -- | 83.0-112.9<br>85.4-110.8 | 2.4-7.2<br>44.8.8 | [27] |
| SPME | Aluminum wire with MIP layer | 3 | GC-MS | 12 | 27-74 | -- | 94.4-99.8 | 4.5-7.4 | [28] |
| SPME | Graphene coated steel wire | 10 | HPLC-DAD | 30 | 0.2 | -- | 86.0-94.6 | 3.5-4.9 | [29] |
| IN-SPDE | MIP | 9 | GC-FID | | 2.6-42 | -- | 82.1-93.5 | 4.4-9.1 | [30] |
| µ-SPE | Conductive polymers | 10 | GC-MS | 40 | 0.01-0.04 | -- | 50-85 | 4.5-9.3 | [31] |
| Ba-µE | ACs | 10 | HPLC-DAD | 960 | 0.091-0.107 | -- | 97.8-102.7 | <15 | [32] |
| IT-SPME | SWCNTs | 1 | DART-MS | | 0.02-0.4 | 411-832 | 85-106 | 3.1-10.9 | [33] |
| IT-SPME | Poly(MAA-EDMA-SWNT) monolith | 4 | Capillary LC | 30 | 0.005-0.05 | -- | 84-103 | 2-16 | [34] |
| MSPE | Graphene-$Fe_3O_4$-MNP | 250 | HPLC-DAD | 20 | 0.025-0.04 | -- | 89.0-96.2 | 3.4-5.2 | [35] |
| MEPS | PANI nanowires network | 7 | GC-MS | | 0.07-0.3 | 462-742 | 66-103 | 5.3-18.4 | [36] |
| LLSME | Toluene/ MIP | 3 | HPLC | 30 | 0.006-0.02 | -- | 81.7-108.7 | 1.2-9.6 | [37] |
| LLME & HF-MMSPE | $C_6H_5Cl$ / MWCNTs | 15 | HPLC-DAD | 20 | 0.08-0.15 | 86-110 | 86.6-106.8 | 4.6-6.9 | [38] |
| HF | o-SWCNHs | 50 | GC-MS | 15 | 0.05-0.1 | 31-133 | 70-100 | <12.8 | [39] |
| SPMTE | MWCNTs | 15 | Micro LC-UV | 20 | 0.2-0.5 | -- | 95-101 | 6-8 | [40] |
| Effervescence- assisted MWCNTs dispersión | MWCNTs | 100 | UPLC-DAD | 3 | 0.15-0.4 | -- | 63-109 | <9.3 | [41] |
| SFPSE | Fabric phase extraction media | 100 | UPLC-DAD | 60 | 0.08-0.47 | 444-1411 | 75-126 | 1.4-11.8 | This method |

[a]Acronyms: SPE, solid phase extraction; IN-SPDE, inside-needle solid phase dynamic extraction; Ba-µE, bar adsorptive micro-extraction; IT-SPME, in tube solid phase microextraction; MSPE, magnetic solid phase extraction; MEPS, microextraction in packet syringe; LLSME, liquid-liquid-solid microextraction; LLME, liquid-liquid microextraction; HF-MMSPE, hollow fiber microporous membrane solid phase microextraction; SPMTE, solid phase membrane tip extraction; MWCNTs, multi-wall carbon nanotubes; SFPSE, stir fabric phase sorptive extraction.
[b]Acronyms: CW, carbowax; DVB, divinylbenzene; PDMS, polydimethylsiloxane; MIP, molecularly imprinted polymer; ACs, activated carbons; SWCNTs, single-wall carbon nanotubes; poly(MAA-EDMA-SWNT), poly(methacrylic acid-co-ethylene dimethacrylate-co-single wall carbon nanotubes); PANI, polyaniline; MNP, magnetic nanoparticles; o-SWCNHs, oxidized single-wall carbon nanohorns.
[c]Acronyms: GC, gas chromatography; MS, mass spectrometry; HPLC, high performance liquid chromatography; UV, ultraviolet; FID, flame ionization detector; DAD, diode array detection; DART, direct analysis in real-time; LC, liquid chromatography; UPLC, ultra performance liquid chromatography.
[d]LODs, limits of detection.
[e]EFs, enrichment factors.
[f]RSD, relative standard deviation.

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## SUPPLEMENTARY MATERIAL

Effect of the ionic strength on the extraction of the target analytes

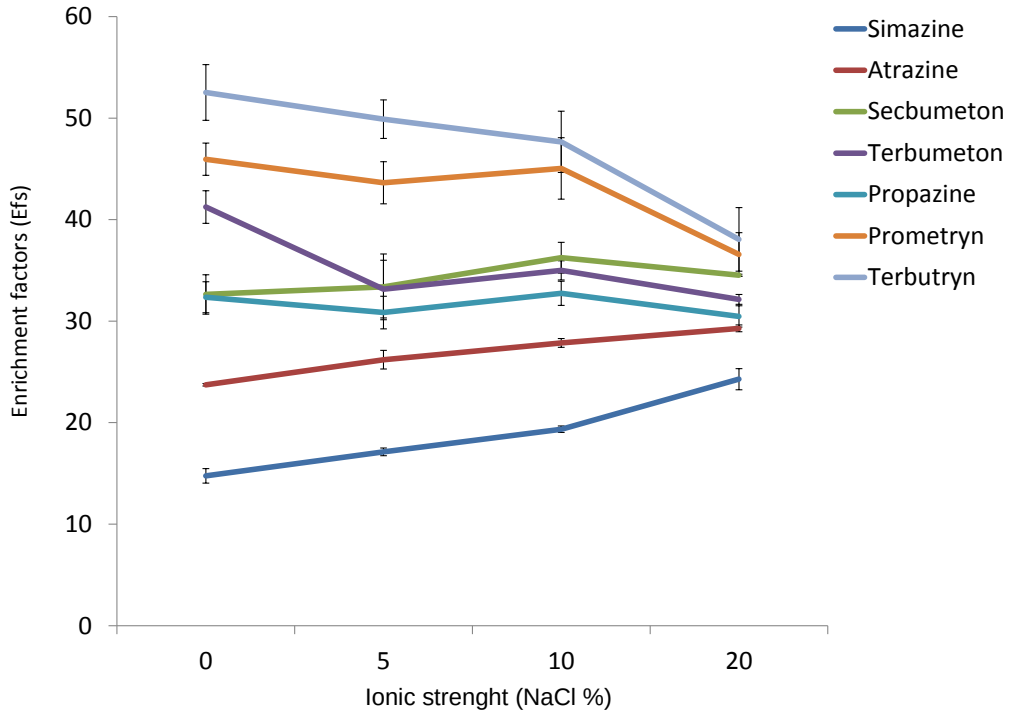


**Figure S1.** Variation of the extraction recovery of the analytes with the ionic strength of the sample. Sodium chloride was employed as model electrolyte

Bivariant effect of sample volume and stirring rate on the enrichment factors for the target analytes

For brevity, the manuscript only presents the data obtained for prometryne which is selected as representative analyte. Although all the analytes presented similar behaviors, the optimum conditions are slightly different for all of them. In the following figures, the data obtained for all the analytes assayed are presented.

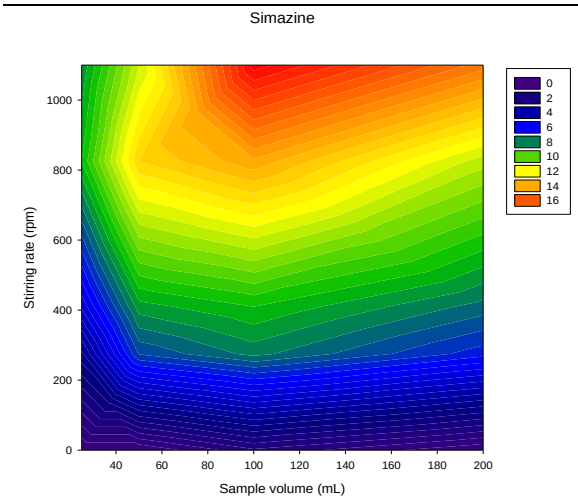


**Figure S2.** Bivariant effect of the sample volume and stirring rate on the analytical signal obtained for a standard solution containing simazine at 50 µg/L.

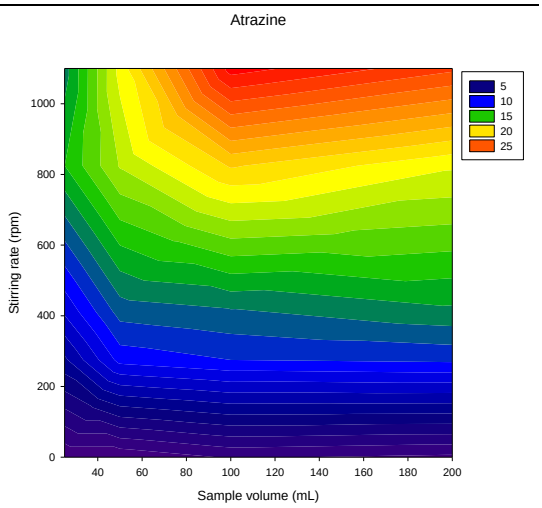


**Figure S3.** Bivariant effect of the sample volume and stirring rate on the analytical signal obtained for a standard solution containing atrazine at 50 µg/L.

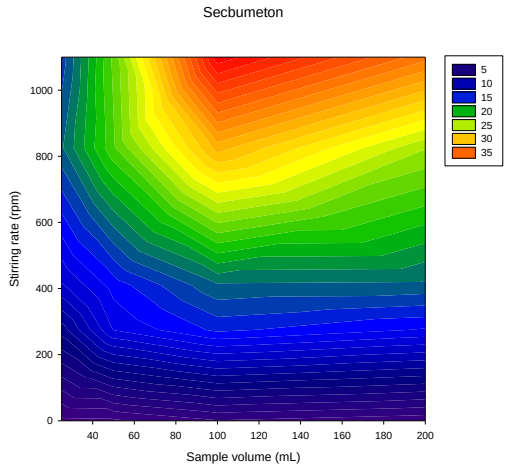


**Figure S4.** Bivariant effect of the sample volume and stirring rate on the analytical signal obtained for a standard solution containing secbumeton at 50 µg/L.

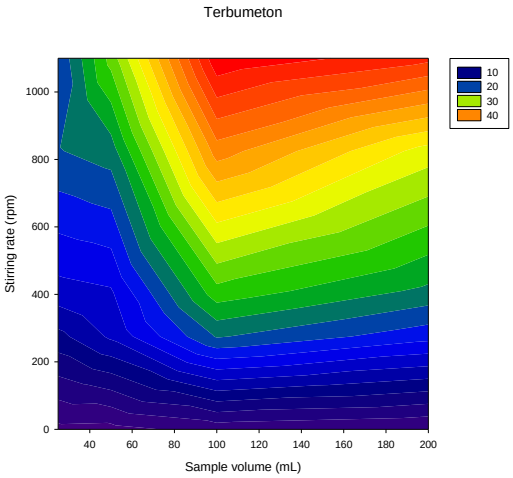


**Figure S5.** Bivariant effect of the sample volume and stirring rate on the analytical signal obtained for a standard solution containing terbumeton at 50 µg/L.

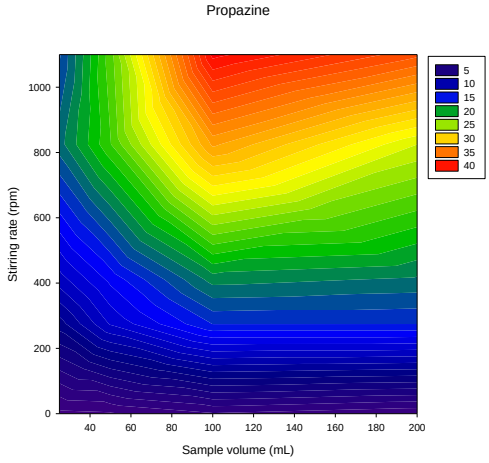


**Figure S6**. Bivariant effect of the sample volume and stirring rate on the analytical signal obtained for a standard solution containing propazine at 50 µg/L.

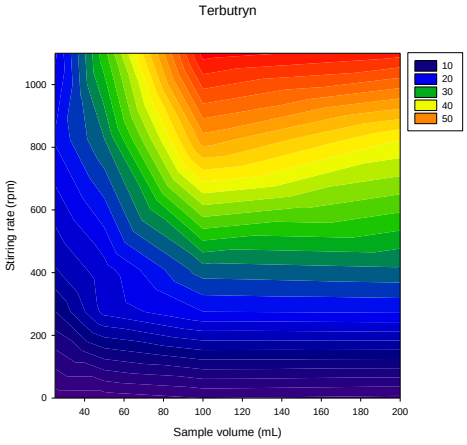


**Figure S7.** Bivariant effect of the sample volume and stirring rate on the analytical signal obtained for a standard solution containing terbutryn at 50 µg/L.

Optimization of the elution process

The elution of the analytes after their retention in the fabric phase sorptive material is essential in order to perform the final chromatographic analysis. Several aspects were optimized, including the elution mode (**Figure S8**), the elution volume (**Figure S9**) and the elution time (**Figure S10**).

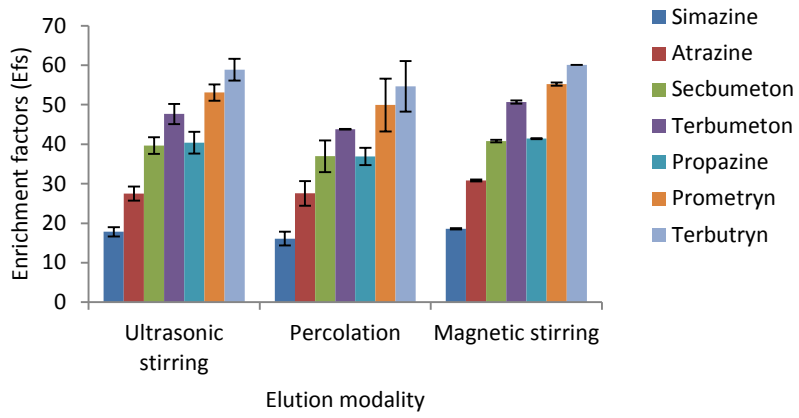


**Figure S8**. Effect of the elution modality on the extraction process.

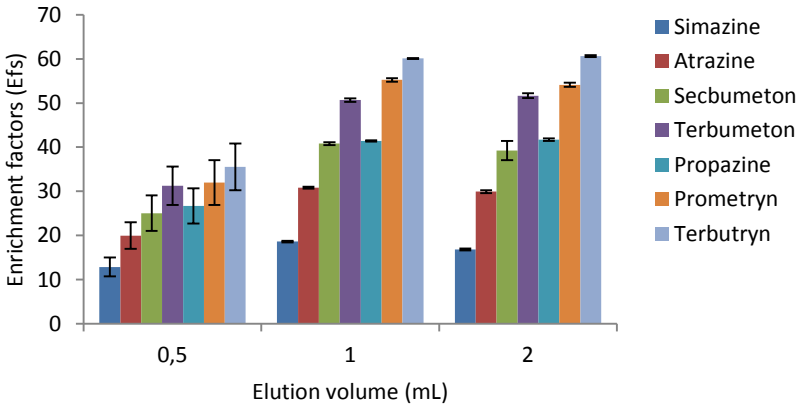


**Figure S9**. Effect of the elution volume on the extraction process.

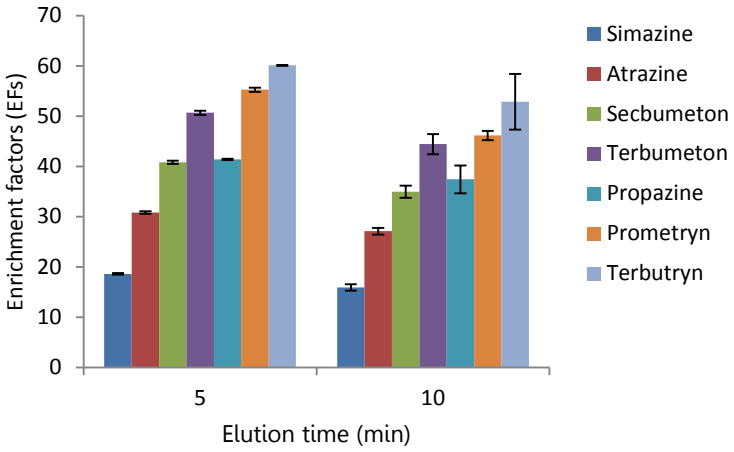


**Figure S10**. Effect of the elution time on the extraction process.

CAPÍTULO 5

# Stir fabric phase sorptive extraction for the determination of benzophenone-type filters in urine

# Stir Fabric Phase Sorptive Extraction for the determination of benzophenone-type filters in urine

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The determination of four benzophenone(BP)-type (BP-1, BP-3, BP-8 and 4-OH-BP) in urine samples, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) is proposed. For this purpose, urine specimens were deconjugated with an acidic treatment and extracted by the stir sol-gel PEG coated fabric phase sorptive extraction (SFPSE) procedure for the measurement of total concentration (i.e., free + conjugated forms) of target analytes. The limits of detection (LODs) were in the range 0.19-0.42 ng/mL and limits of quantification (LOQs) were 0.63-1.38 ng/mL. The precision, expressed as relative standard deviation (RSD) calculated at a concentration of 2 ng/mL ranged from 4.9 % to 11.3% (n=5). Absolute extraction recoveries were between 15-17%. Finally, the developed method was applied to the determination of selected BPs in human urine samples. Relative recoveries were within 75-109% which demonstrated the applicability of the proposed method to the selected analytical problem.

***Keywords***: *Stir fabric phase sorptive extraction (SFPSE); Benzophenones; Urine sample; Microextraction; Liquid chromatography.*

## 1. INTRODUCTION

Benzophenones (BPs) are aromatic ketones that are widely employed in the formulation of sunscreens protecting the skin from UVA and UVB rays [1]. 2- Hydroxy-4-methoxybenzophenone (benzophenone-3 or BP-3) is by far the most frequently used UV-filter to formulate spray sunscreen lotions. BPs are considered endocrine disrupting chemicals (ECD) and they can interfere in the normal functioning of the endocrine system [2-3] as it has been demonstrated in many in-vitro and in-vivo studies [4-5]. For this reason, the European Union (EU) Regulation 1223/2009 stipulates the compounds that are able to be used as UV-filters in cosmetics and their maximum concentrations [6]. As it can be inferred, dermal adsorption is the main route of exposure to BPs [7]. BPs can both accumulate and be excreted. On the one hand, free forms of EDCs can accumulate in certain human tissues such as placental tissue [8] and human milk [9] due to their lipophilic nature producing harmful disrupting effects. On the other hand, BPs can also be conjugated with β-d-glucuronide and sulphate, reducing their bioactivity and facilitating their urinary excretion because of their high water solubility. In order to release the free BP from these glucuronides, urine samples are usually hydrolyzed before its treatment. This hydrolysis can be performed chemically (using alkalis or acids) [10] or biochemically (using enzymes) [11-12]. The former approach is cheaper but induces an intense modification of the sample matrix while the latter is more expensive but it presents a better selectivity. Both approaches have been successfully applied for BPs determination.

Once released from the glucuronides, BPs are usually isolated from the sample matrix and preconcentrated to improve the selectivity and sensitivity of their determination. Classical sample preparation techniques including solid-phase [13] and liquid-liquid extraction [11] have been employed for this aim. In the microextraction realm, hollow fiber liquid phase microextraction [14], stir bar sorptive extraction [15], dispersive liquid-liquid microextraction [16] and single drop microextraction [17] have been proposed in the last years.

Due to the complexity of biological matrices, new sample preparation approaches are needed to ensure effective sample clean-up as well as trace-level determination. In this context, fabric phase sorptive extraction (FPSE) emerged [18]. FPSE, the most recent member of the sorptive microextraction techniques, is based on permeable natural/synthetic fabrics, which can be of different nature (including cotton, polyester, fiber glass). It is used a support where a sorptive phase is coated by sol-gel technology [19]. These phases present several advantages over conventional formats. On the one hand, fabric phases are versatile since different coatings, covering a wide range of polarities, can be employed. On the other hand, the film format makes easier their handling and promotes the extraction of the analytes in an efficient way. The potential of FPSE has been demonstrated by its application to the determination of estrogens in urine [20], amphenicol residues in milk [21] and anti-inflamatory drugs in water samples [22].

A new solid phase microextraction approach, called stir fabric phase sorptive extraction (SFPSE), has been recently proposed [23]. This technique integrates in a dedicated device the sorptive phase and the stirring element allowing the extraction of the analytes in a simple and efficient way. In this case, the SFPSE is practically applied to the extraction of BPs from urine samples. All the variables involved in the extraction process have been considered in depth and the methodology has been characterized in terms of linearity, sensitivity, precision and accuracy.

## 2. EXPERIMENTAL SECTION

### 2.1. Materials and reagents

The reagents used were of analytical grade or better. Sol-gel active polymer poly(ethylene glycol) was purchased from AlfaAesar (Ward Hill, MA, USA). Acetone, dichloromethane, methyltrimethoxysilane (MTMS), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Sodium hydroxide and hydrochloric acid were purchased from

Thermo Fisher Scientific (Milwaukee, WI, USA). Substrates for fabric phase sorptive extraction media were obtained from Jo-Ann Fabric (Miami, FL, USA). For sol-gel PEG coating, 100% cotton cellulose fabric was used as the substrate. The four benzophenone type selected: benzophenone-1 (BP-1), benzophenone-3 (BP-3), benzophenone-8 (BP-8) and 4-hydroxy-benzophenone (4-OH-BP) were purchased from Sigma–Aldrich (Madrid, Spain). Stock standard solutions of each analyte and internal standard were prepared in methanol (Sigma–Aldrich) at a concentration of 1 g/L. Working solutions were prepared on a daily basis by rigorous dilution of the stocks in Milli-Q water (Millipore Corp., Madrid, Spain) or methanol as required.

Hydrochloric acid (36%) and ammonia (25%), both from Panreac, were used for pH adjustment. Synthetic urine was purchased from Sigma-Aldrich and was employed as analytes-free urine samples.

### 2.2. Preparation of FPSE media

The preparation of sol-gel poly(ethylene glycol) (PEG) coated fabric phase sorptive extraction media consist of three and well defined steps: (1) pretreatment of fabrics for sol-gel coating, (2) preparation of the sol solutions for coating the substrate and (3) formation of sol-gel PEG coating on the substrate. The synthesis is described in detail elsewhere [23].

### 2.3. Sample preparation

Urine samples (5 mL) were collected from a healthy woman and stored at -18 ºC until the time of sample pretreatment. Prior to analysis, urine samples were hydrolyzed with concentrate HCl thermostated at 80ºC for 1h. Later on, the pH of the urine sample was neutralized with concentrate aqueous ammonia hydroxide. Urine samples were diluted 1:9 (v:v) with distilled water prior to be subjected to the SFPSE procedure. Blank samples were analyzed in order to ensure the absence of analytes or that these were below the limit of detection (LODs) of the method.

### 2.4. Chromatographic analysis

Two chromatographic systems, including different detectors, were employed in the development of the present research. This strategy avoids the introduction of co-extracted matrix components on the mass spectrometer which is especially critical when a new extraction procedure is being developed. Once the extraction was characterized and the potential presence of co-extracted compounds evaluated, MS can be safely used for method validation.

The optimization of the extraction procedure was carried out on a Waters-AcquityTM Ultra Performance LC system (Waters Corp., Madrid, Spain) using an Acquity UPLC® BEH $C_{18}$ column (1.7 µm particle size, 2.1 mm × 50 mm) maintained at 45 ºC. The mobile phase consisted of (A) water and (B) acetonitrile at a flow rate of 0.5 mL/min using a gradient elution program. The initial composition was fixed at 60 % A, the percentage being decreased to 40 % in 5 min. The injection volume was 5 µL with partial loop with needle overfill mode. The separated analytes were determined using a PDA eλ (extended wavelength) Detector (Waters) at 313 nm. System control was achieved with Empower software.

The validation of the methodology was performed on an Agilent series 1260 HPLC system equipped with a pump, a degasser, an autosampler and a thermostated column compartment (AgilentTechnologies, Palo Alto, CA, USA). Chromatographic separation was carried out at 40 ºC on an Agilent Poroshell 120 SB-$C_{18}$ analytical column (2.1 mm × 75 mm, 2.7 µm). The mobile phase comprised 0.1% formic acid aqueous solution (solvent A) and LC-MS grade methanol with 0.1% formic acid (solvent B) at a flow rate of 0.2 mL/min and it was operated under the gradient elution mode. The gradient program was as follows: 0-3.5 min, 60% B, 3.5-10 min, 60-95% B and back to 60%B in 0.5 min. Flow rate was set at 0.2 mL/min. Equilibration time was fixed in 9 min. 50 µL was selected as injection volume.

MS spectra analyses were conducted on an Agilent 6420 triple quadrupole mass spectrometer equipped with electrospray ionization source (Agilent Corporation, MA, USA). Quantification was performed in positive using multiple reaction monitoring (MRM). Instrument parameters were as follows: source temperature, 350 ºC; cone gas flow, 9 L/min; capillary voltage, 2500V, nebulizer gas flow, 60 psi and cell accelerator voltage 7V. Nitrogen (99.995%) was used as cone, desolvation and collision gas. Dwell time was set at 200 ms. Optimized parameters for each compound are also listed together with the mass transitions in **Table 1**. Data acquisition was performed with MassHunter Workstation (Agilent Technologies, USA).

**Table 1.** Transitions and optimized potentials for LC–MS/MS analysis.

| Compound | Transitions | Fragmentor (V) | CE (V) |
|---|---|---|---|
| BP-1 | 214.9 → 136.9[a] | 106 | 13 |
| | 214.9 → 104.9[b] | 106 | 15 |
| BP -3 | 229.0 → 150.9[a] | 118 | 19 |
| | 229.0 → 104.9[b] | 118 | 15 |
| BP -8 | 244.8 → 120.9[a] | 110 | 11 |
| | 244.8 →150.8[b] | 110 | 12 |
| 4- OH-BP | 198.8 → 121[a] | 104 | 15 |
| | 198.8 → 105[b] | 104 | 15 |

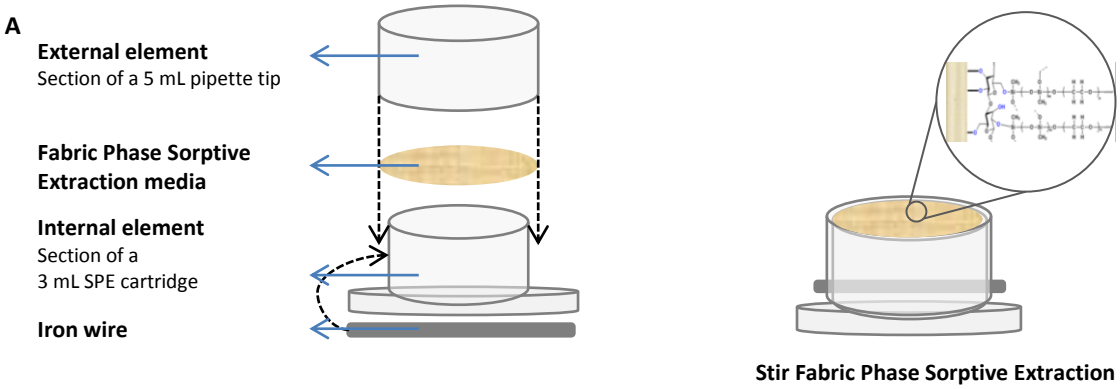
[a]MRM transition used for quantification.
[b]MRM transition used for confirmation.

### 2.5. SFPSE procedure

SFPSE, as it is described elsewhere [23], was accomplish utilizing four elements. The set-up used in the experiments is shown in **Figure 1A**. The fabric phase was fixed by press-fit

(squeezed) between the internal and the external circular element. Once the fabric phase is sealed, the extraction device is pierced with an iron wire to allow its magnetic stirring.

The scheme describing the SFPSE procedure is shown in **Figure 1B**. First, 50 mL of sample was loaded in the beaker. The samples were either urine or aqueous standards of the four BPs in pure water. Second, the SFPSE, previously conditioned with methanol, is introduced in the beaker and stirred at 1100 rpm for 45 min to carry out the extraction of the target analytes. Once finished, the SFPSE unit is withdrawn from the solution and immersed faced down in 1 mL of methanol (only the fabric phase being in close contact with the solvent) and keep under magnetic stirring for 5 min for analytes elution. Finally, the eluted solvent containing the target analytes was filtered through a disposable syringe filter (Nylon, 0.45 μm) to obtain particulate-free solution. To improve the sensitivity of the measurements, the extract is evaporated to dryness under a nitrogen stream. For UPLC-DAD analysis the final residue was re-dissolved in 50 μL of methanol while for LC-MS/MS analysis, the residue was re-dissolved in 50 μL of methanol which was subsequently mixed with 50 μL of a 0.1% (v/v) formic acid aqueous solution to improve the chromatographic separation. Between extractions, the stir fabric phase sorptive extraction unit is magnetically stirred in 2 mL of methanol to avoid any potential carry-over effects.



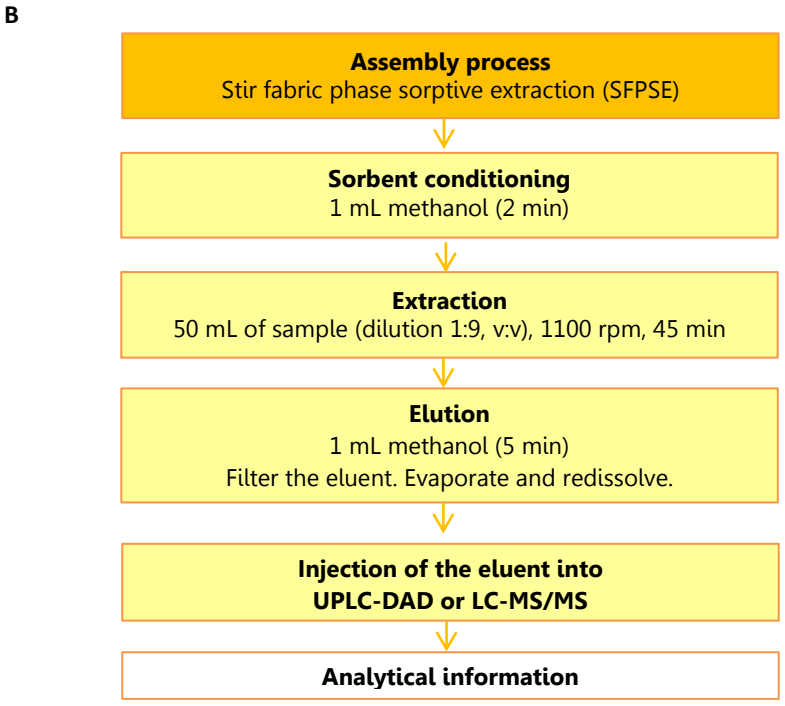




**Figure 1.** Schematic representation of (A) SFPSE set-up and (B) steps involved in SFPSE process.

## 3. RESULTS AND DISCUSSION

### 3.1. Selection of the variables affecting to the SFPSE procedure

Different variables, which are summarized in **Table 2**, may affect the efficiency of the extraction procedure and therefore their effects on the analytes extraction were considered in depth. **Table 2** also reflects their initial values, the interval studied and the optimum values for each variable. The optimization was performed under a univariate approach although a couple of variables (sample volume and the stirring rate) were considered jointly since they are directly related. Initially, an aqueous standard solution containing the benzophenones at a concentration of 50 ng/mL was used in these studies with UPLC-DAD.

**Table 2.** List of the variables involved in the SFPSE process.

| Variable | Initial value | Interval studied | Selected value |
|---|---|---|---|
| Sample pH | | 3-10 | 3-7 |
| Ionic strength (g/L NaCl) | 0 | 0 - 200 | 0 |
| Sample volume (mL) | 50 | 15 - 50 | 50 |
| Stirring rate (rpm) | 1100 | 0 - 1100 | 1100 |
| Extraction time (min) | 15 | 5-120 | 45 |
| Elution solvent | Methanol | Methanol, acetonitrile, acetone | Methanol |
| Elution modality | Magnetic stirring | Ultrasound, magnetic stirring, percolation | Magnetic stirring |
| Elution volume (mL) | 1 | 0.5-2 | 1 |

#### *3.1.1. Effect of the pH*

In order to study the effect of the pH of the sample in the extraction performance, the pH was adjusted to 3, 4, 7 and 10 by using 1M HCl or 1M NaOH solutions. The samples were analyzed by the SFPSE methodology. The signals remained constant for all analytes when pH was between 3 and 7, decreasing dramatically at pH 10. This finding is in good

concordance with the dissociation constant values of the analytes. For this reason, optimum pH for extraction of benzophenones was selected in the range from 3 to 7.

### *3.1.2. Effect of addition of salt*

Ionic strength may play a key role in microextraction techniques. The salting-out effect was investigated with different NaCl concentration in the range from 0 to 200 g/L. In general, the peak areas slightly increase with increasing salt concentration in the aqueous sample up to 100 g/L, but the extractions were much more irreproducbly (probably due to the higher viscosity of the media). Consequently, NaCl was not added to the sample for further studies.

### *3.1.3. Effect of the stirring rate and sample volume on the extraction*

The sample volume and the stirring rate are related variables. Generally, when higher samples volumes are processed, higher stirring rates can be used. Additionally, this strong stirring induced convection in the sample, which was important for the mass transfer to the sorbent material. Both variables were optimized together considering three different sample volumes (15, 25 and 50 mL) and five different stirring rates (0, 410, 550, 825 and 1100 rpm). **Figure 2** shows the bivariant effect on the extraction (EFs) by means of a contour surface graph. As seen from the graphical, the maximum enrichment factors were obtained when large samples volumes were employed and high stirring rates. For this reason, 50 mL and 1100 rpm were chosen.

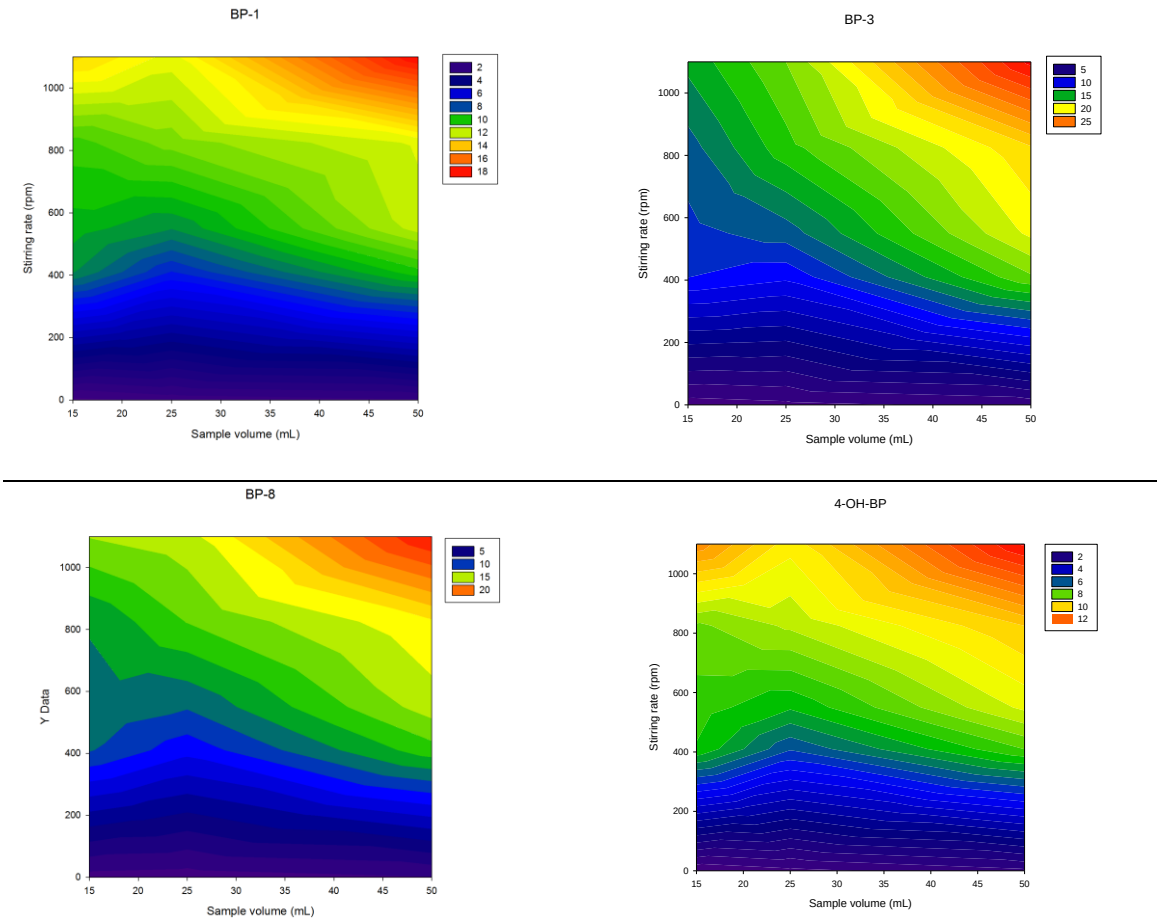



**Figure 2**. Bivariant effect of the sample volume and stirring rate in the analytical signal obtained for a standard solution at 50 ng/mL.

### *3.1.4. Effect of the extraction time*

As other solid phase microextraction procedures, the proposed extraction technique is time dependent. In a subsequent experiment, the extraction time was varied between 5 and 120 min. Enrichment factors versus extraction time are plotted in **Figure 3**. The enrichment factors increased with longer extraction times up to approximately 45 min, whereas no

further increased in extraction performance was observed. Therefore, 45 min was accomplished during the rest of this work.

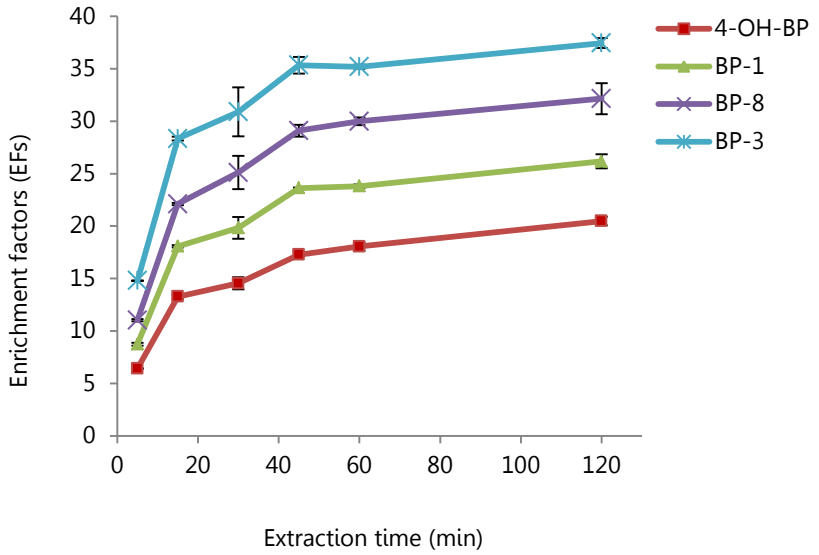



**Figure 3.** Effect of the extraction time on the enrichment factors of the benzophenones.

### *3.1.5. Elution process*

Once the analytes have been conveniently extracted, they should be eluted for the subsequent chromatographic analysis. Three different eluents were tested, namely: methanol, acetonitrile and acetone, being methanol selected since it provided the best results in terms of sensitivity and precision. Three different elution strategies, namely: ultrasonic stirring, magnetic stirring and elution by percolation, were evaluated. According to the sensitivity and precision of the different modes, magnetic stirring was selected as the best approach. The elution volume was studied in the range from 0.5 to 2 mL, 1 mL being selected as the optimum value. In order to improve the sensitivity, the final extract was evaporated under a $N_2$ stream and redissolved in 50 µL of methanol or 50 µL of methanol +

50 µL of 0.1 % v/v formic acid aqueous solution depending on the chromatographic separation configuration used.

### 3.2. Evaluation of the matrix effect

In a first of experiment, 50 mL of urine sample (spiked with the BPs to a concentration of 10 ng/mL) was analyzed with the proposed methodology. During the extraction procedure, the fabric phase was dyeing and the extracts obtained after elution were yellow, which clearly evidence an important matrix effect. The extracts were filtered and analyzed by LC-MS/MS, obtaining lower recoveries from urine than from pure water. In fact, the application of microextraction analytical techniques to biological samples can be translated into a lower enrichment factors and thus lower absolute recoveries. On the one hand, other component of the matrix can compete with analytes for the sorbent, altering the distribution coefficient and decreasing the extraction efficiency or slowing down extraction kinetics. On the other hand, low extraction recoveries are usually ascribed to protein binding.

In a second experiment, a clean-up step was introduced after extraction and before the chemical elution. Three different clean-up solutions were tested, namely: i) water, ii) water: methanol (80:20, v/v) and iii) 0.1 % (v/v) trifluoroacethic acid solution. This additional step provides cleaner extracts, however, no enhancement in the extraction recoveries was observed. The same occurs when introducing both a centrifugation step and filtering (Nylon, 0.45 µL) before the extraction.

In another experiment, a solid phase extraction (SPE) step was introduced before SFPSE aimed to remove interfering matrix constituents. For this purpose, C2 cartridge was employed. First of all, the cartridge was conditioned with methanol and water. 50 mL of spiked urine was adjusted to pH 12 (at basic pH the analytes are not retained in the sorbent) and passed through the sorbent. Later on, the sample was acidified to pH 3 and extracted by SFPSE. No further gained in the extraction recoveries was observed.

As it is well known that matrix effects can be reduced by sample dilution, proper aliquots of urine were taken and diluted at different ratios (1:1, 1:4 and 1:9) with an aqueous standard containing all the analytes at different concentration in order to maintain constant the analyte concentration in the final dilution (the sample volume was fixed at 50 mL). Higher dilutions reduce or eliminate matrix effects more successfully; in fact the extraction efficiency obtained 1:9 are slightly superior in comparison with dilution 1:1. On the other hand, better sensitivity is required as dilution factors increase. If detection sensitivity is not an issue, dilution of the sample from any kind of biological material is a good trick to reduce the matrix problems and increase the repeatability/precision of the method. For the above reasons, urine samples were diluted 10 times prior the extraction process and with this dilution, no matrix effects were found.

### 3.3. Analytical figures of merit and recovery study

Once the proposed procedure was optimized, it was evaluated for the determination of the target benzophenone-type in urine samples. The calibration curves for the target analytes were constructed in the range of 0.25-50 ng/mL by using five diluted synthetic urine samples (1:9, v/v) where each of the samples were spiked with the four target analytes at controlled concentration levels.

As seen from **Table 3**, good linearity was obtained with $R^2 \geq 0.997$. The sensitivity of the method was evaluated according to the limits of detection (LOD) and quantification (LOQs). The LODs, which were calculated using a signal to noise ratio of three ranged from 0.19 ng/mL to 0.42 ng/mL while the LOQs, calculated using a signal to noise ratio of ten, varied between 0.63 ng/mL and 1.38 ng/mL. The precision expressed as relative standard deviation (RSD) in synthetic urine at 2 ng/mL (n=3) was between 4.9-11.3%. The enrichment factors obtained from synthetic urine were in the range from 5 to 9, which involves an absolute extraction recovery of 15-17%. Low absolute recoveries are not a disadvantage as long as the relative recovery, precision and reproducibility of the method are high.

The proposed method was applied for the determination of the target benzophenones in human urine samples. First, an aliquot of urine was subjected to the deconjugation step previously described, and then diluted 1:9 (v/v). The samples were analyzed by the proposed SFPSE procedure to find any potential presence of the analytes. As no positive samples were found, a recovery study was performed. Aliquots of urine were spiked at 10 ng/mL concentration level, subjected to the whole analytical processes and analyzed in quintuplicate. The corresponding relative recovery data are reported in **Table 3** and were between 75-109 %.

The evaluation data supported that future method development based on the principles of SFPSE can be expected to provide reliable data when combined with LC-MS/MS. However, to fully validate the method under the FDA guideline [24], a complete validation, including precision measured inter-day and intra-day as well as a complete study of the recovery at three different concentration level (low, medium and high), should be performed in the very near future.

**Table 3**. Figures of merit of the proposed SFPSE procedure.

| Analytes | Lineal range (ng/mL) | $R^2$ | LOD[a] (ng/mL) | LOQ[b] (ng/mL) | RSD[c] at 2 ng/mL (n=3) | EF[d] | AER[e](%) | R[f] ± SD |
|---|---|---|---|---|---|---|---|---|
| BP-1 | 0.25-50 | 0.995 | 0.25 | 0.82 | 4.9 | 7 | 15 | 75 ± 8 |
| BP-3 | 0.25-50 | 0.993 | 0.19 | 0.63 | 10.8 | 8 | 17 | 95±9 |
| BP-8 | 0.25-50 | 0.992 | 0.22 | 0.73 | 11.3 | 8 | 17 | 89 ± 7 |
| 4-OH-BP | 0.25-50 | 0.994 | 0.42 | 1.38 | 9.6 | 5 | 11 | 109 ± 10 |

[a]LOD limit of detection.
[b]LOQ limit of quantification.
[c]RSD relative standard deviation.
[d]EF enrichment factor.
[e]AER absolute extraction recovery.
[f]R relative recovery calculated at 10 ng/mL.

## 4. CONCLUSIONS

Stir fabric phase sorptive extraction (SFPSE), which integrates sol-gel hybrid organic-inorganic coated fabric phase sorptive extraction media with a magnetic stirring mechanism, is presented for the first time for the analysis of biological samples.

Prior to the extraction, urine specimens were deconjugated by means of an acidic treatment in order to quantify free and conjugated forms. The isolation and preconcentration of analytes from the samples was properly optimized. The combination of SFPSE with LC-MS/MS for the analysis of four benzophenone (BP-1, BP-3, BP-8 and 4-OH-BP) from urine samples have provided LODs at low ng/mL with good precision (≤11.3 %). Although the number of analyzed samples is not very high, it is possible to formulate some interesting conclusions.

First, the proposed methodology may be useful for the determination of trace levels of BPs in urine samples, since limits of quantification ranged between 0.63-1.38 ng/mL and therefore, it may be used to perform biomonitoring studies. Second, the current SFPSE reports shorter equilibrium extraction time (45 min) in comparison with FPSE (100 min) [22], which is ascribed to the beneficial effect of integrating extraction and stirring in the same device. Finally, the simplicity in design, easy to use, portability, and wide range of readily available sol-gel based sorbents with tunable selectivity and porosity have made SFPSE a highly promising technique.

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BLOQUE IV

# MICROEXTRACCIÓN EN FASE LÍQUIDA CON UNIDADES AGITADAS

**Capítulo 6**

Stir octadecyl-modified borosilicate disk for the liquid phase microextraction of triazine herbicides from environmental waters.

**Capítulo 7**

Stir membrane liquid microextraction for the determination of paracetamol in human saliva samples.

**Capítulo 8**

Parallel artificial liquid membrane extraction of acidic drugs from human plasma.

La extracción líquido-líquido convencional (*liquid-liquid extraction*, LLE) es una de las técnicas más empleadas para la limpieza de muestras y preconcentración de compuestos de interés. A pesar de ser una técnica muy versátil, el empleo de un gran volumen de muestra y de disolventes, la facilidad con la que se forman emulsiones, el tiempo necesario y la obtención de factores de preconcentración bajos (a no ser que se lleven a cabo procesos de evaporación y reconstitución) en comparación con otras técnicas, hacen de la LLE una técnica lenta, cara y poco respetuosa con el medio ambiente y el operador. Como respuesta a la necesidad de superar estas limitaciones, surgió la técnica de microextracción en fase líquida (*liquid phase microextraction*, LPME). Esta es simple, rápida y de bajo coste ya que el volumen de disolvente utilizado se reduce a los pocos microlitros. Desde su origen, ha experimentado una evolución continua, dando lugar a una gran variedad de modalidades.

El objetivo fundamental de este Bloque de la Memoria es el desarrollo y aplicación de nuevas modalidades de microextracción en fase líquida como herramientas en el pretratamiento de muestras líquidas de origen ambiental y biológico.

En primer lugar y con respecto al análisis de muestras ambientales, en el **Capítulo 6** se han utilizado discos de borosilicato como herramientas para el desarrollo de metodologías analíticas que permitan el aislamiento y la preconcentración de triazinas en aguas de río. Para ello, se inmovilizaron en los poros del disco cadenas octadecilo ($C_{18}$). Estas cadenas de $C_{18}$ se utilizaron para estabilizar a través de interacciones hidrofóbicas al disolvente orgánico de naturaleza no polar, concretamente se utilizó tolueno, siendo este el responsable de la extracción. Para favorecer la cinética del proceso de extracción, se diseñó un cuerpo de plástico con un imán incorporado sobre el cual se depositó el disco, haciendo posible su agitación magnética. La separación y detección de los analitos se llevó a cabo mediante cromatografía líquida de ultra alta presión acoplada a detector de diodos en fila ultravioleta/visible. En este trabajo se realiza también el análisis DAFO (debilidades,

amenazas, fortalezas y oportunidades) de la técnica presentada, así como la comparación directa con otras metodologías existentes para la resolución del mismo problema analítico.

En segundo lugar, se presentan dos técnicas de microextracción en fase líquida (en ambas se trabajó en la modalidad de tres fases) en el ámbito del bioanálisis. En cuanto a la naturaleza de las muestras biológicas, se analizaron biofluidos, en concreto se optó por muestras de saliva y de plasma. Como problema analítico se seleccionaron distintos fármacos en base a su amplio uso, así como por su estructura y propiedades físico-químicas. El primer sistema de extracción propuesto, descrito en el **Capítulo 7**, se ha adaptado la técnica de microextracción líquida con membrana agitada que normalmente requiere volúmenes de muestra de 15 a 50 mL, al análisis de muestras de disponibilidad limitada. Se llevó a cabo la determinación de paracetamol en saliva y se hizo un estudio farmacocinético. La validación de la metodología propuesta se realizó de acuerdo a los parámetros establecidos en la guía FDA, U.S. (*Food and Drug Administration*). Para el análisis se utilizó cromatografía de líquidos con detección ultravioleta-visible.

En el **Capítulo 8** se aplica por primera vez la microextracción con membrana líquida artificial en paralelo a la determinación de compuestos exógenos de naturaleza ácida (anti-inflamatorios) en muestras de plasma mediante cromatografía de líquidos con detección de diodos en fila ultravioleta/visible. Lo más destacable de esta metodología es que permite realizar hasta 96 extracciones de manera simultánea. Debido a sus características, se plantea la posibilidad de su automatización completa en investigaciones futuras.

CAPÍTULO 6

# Stir octadecyl-modified borosilicate disk for the liquid phase microextraction of triazine herbicides from environmental waters

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# Stir octadecyl-modified borosilicate disk for the liquid phase microextraction of triazine herbicides from environmental waters

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In the present article, a novel extraction/stirring approach in the liquid phase microextraction context is presented. The new technique is based on octadecyl coated borosilicate disks which act as support of the organic extracting solvent thanks to hydrophobic interactions. The disk is integrated in a stirring element which favors the transference of the analytes to the extraction phase. The proposed extraction procedure has been characterized using the determination of nine herbicides in water samples by ultra performance liquid chromatography (UPLC) combined with ultraviolet (UV) detection as model analytical problem. All the variables involved in the extraction have been studied and optimized in depth and the optimized technique provides enrichment factors in the range from 79 to 839. The method has been analytically characterized on the basis of its linearity, sensitivity and precision. Limits of detection were in the range from 0.14 µg/L (atrazine) to 0.56 µg/L (terbumeton) with precision better than 7.3% (expressed as relative standard deviation). Relative recoveries were close to 100%, which demonstrated the applicability of the stir octadecyl-modified borosilicate disk for the proposed analytical problem.

***Keywords:*** *Liquid phase microextraction; Stir octadecyl-modified borosilicate disk; Extraction/stirring integrated techniques; Triazines; Water samples.*

## 1. INTRODUCTION

Many official methods of analysis still use liquid-liquid extraction (LLE) [1] and solid phase extraction (SPE) [2] as sample treatment. Despite their usefulness, these techniques are being substituted by novel extraction procedures which improve the enrichment factors and reduce the solvent consumption [3]. Among the novel sample treatment procedures, extraction and stirring integrated techniques have been in a continuous evolution [4] since 1999, when stir bar sorptive extraction (SBSE) was presented [5]. The stirring of the sample during the extraction accelerates the analytes diffusion from the bulk solution to the extraction interface reducing the Nernst boundary layer thickness.

The design of novel extraction/stirring integrated devices has become a promising research field in order to face up the main limitations of the classical technique, specially related to the coatings and their mechanical instability. In this context stir membrane extraction (SME) [6, 7] and rotating-disk sorptive extraction (RDSE) [8-12], both proposed in 2009, can be considered promising alternatives. SME is based on the use of a polymeric membrane as extracting phase while RDSE uses a thin film of polydimethylsiloxane.

In the same context, stir rod sorptive extraction [13] and stir cake extraction [14] have been proposed to extend the applicability of monolithic materials in extraction/stirring integrated techniques. In fact, the cracking tendency of monoliths is overcome by avoiding their direct contact with the vessel walls or by protecting them with a plastic body. In addition, stir frit microextraction [15] exploits the inherent extractive capabilities of commercial polyethylene frits which are pierced with an iron wire to allow its stirring.

In the liquid phase microextraction (LPME) realm, the so-called dual solvent-stir-bar [16] was the first technique to accomplish the extraction and stirring integration. In the same context, Es'haghi *et al*. proposed carbon nanotubes-assisted pseudo-stir-bar solid-liquid microextraction [17] which is an hybrid between liquid and solid phase microextraction. Stir membrane liquid phase microextraction (SM-LPME) [18] tried to improve the extraction

capacity of SME using a solvent, instead of a membrane, as extracting phase. The original approach was based on two-phase mode which is especially applicable to the extraction of non-polar compounds from aqueous samples. The main drawback of this approach was the stabilization of the solvent in the extraction unit which limits the extraction time and specially the sample volume that can be processed. SM-LPME can be also performed in a three-phase mode [19-21] which presents a great potential for the extraction of ionisable non-polar compounds.

In this paper, a novel microextraction technique called stir octadecyl-modified borosilicate disk is presented. The new technique, which operates under the LPME principles, shares some building elements with SME. However, the core of the unit is based on the use of commercial bare borosilicate disks coated with octadecyl ($C_{18}$) groups. The $C_{18}$ groups allow the stabilization by hydrophobic interactions of a non-polar solvent (extractant) on the disk pores. These interactions are intense enough to maintain the solvent in the disk despite the high stirring rates employed which is a clear advantage over two-phase SM-LPME. The novel approach has been practically evaluated using the determination of nine triazines in water by UPLC/DAD as model analytical problem.

Triazines are used as herbicides for the control and elimination of broad leaf and grassy weeds in a wide range of agricultural crops. These herbicides present a high toxicity, high persistence and accumulation in the environment even when they are used at very low concentrations due to their high solubility and to their physicochemical properties [22]. For this reason these pollutants should be monitored in natural waters and, taking into account the restrictive maximum allowable levels established by both the US Environmental Agency (EPA) and the European Union (EU), an efficient sample preconcentration step is required.

## 2. EXPERIMENTAL SECTION

### 2.1. Reagents, materials and samples

The reagents used were of analytical grade or better. The nine triazines selected: simazine (SMZ), simetryn (SMT), atrazine (ATZ), secbumeton (SBM), prometon (PMT), terbumeton (TBM), propazine (PPZ), prometryn (PMT) and terbutryn (TBT) were purchased from Sigma–Aldrich (Madrid, Spain). Stock standard solutions of each analyte were prepared in acetonitrile (Sigma–Aldrich) at a concentration of 1 g/L, simazine excepted (500 mg/L), and were prepared on a daily basis by rigorous dilution of the stocks in Milli-Q water (Millipore Corp., Madrid, Spain) or acetonitrile as required. Sodium chloride from Sigma-Aldrich was used to adjust the ionic strength of the standards and samples. Toluene, hexane, ethyl acetate, chloroform and methanol were purchased from Sigma-Aldrich and they were evaluated as extraction solvents.

Borosilicate filter disks (20 mm in diameter and pore size of 10-16 μm) were purchased from ROBU (Glasfilter-Geraete GmbH, Germany). All the reagents required for the synthesis of octadecyl-modified borosilicate disks were purchased from Sigma-Aldrich. Hydrogen peroxide, sulfuric acid were employed to activate the disk for its derivatization while ethanol was used for washing. Finally, trimethoxy(octadecyl)silane and dry toluene were used to introduce hydrophobic groups on the borosilicate disks.

The construction of the stirring devices required a 20-mL plastic syringe purchased from Terumo (Heverlee, Belgium) and a cylindrical magnetic rod (4 mm x 25 mm and 42 N of maximum magnetic force) purchased from Supermagnete (Gottmadingen, Germany). The magnetic rod was protected with a thin layer of polyvinyl chloride (Sigma-Aldrich).

Different environmental brook water samples from Córdoba (I) and Jaén (II and III), Spain, were analyzed. All the samples were collected in amber-glass bottles without headspace and stored in the dark at 4 ºC until their analysis. Prior to the extraction, no filtering was carried out.

### 2.2. Chromatographic analysis

Chromatographic analyses were carried out on a Waters-AcquityTM Ultra Performance LC system (Waters Corp., Madrid, Spain) using an Acquity UPLC® BEH $C_{18}$ column (1.7 μm particle size, 2.1 mm × 100 mm) maintained at 45 ºC. The mobile phase consisted of (A) water and (B) acetonitrile at a flow rate of 0.5 mL/min using a gradient elution program. The initial composition was fixed at 75% A, the percentage being decreased to 40% in 10 min. The injection volume was 5 μL with partial loop with needle overfill mode. The separated analytes were determined using a PDA eλ (extended wavelength) Detector (Waters) at 223 nm. System control was achieved with Empower software.

### 2.3. Synthesis of the octadecyl-modified borosilicate disk and its coupling to the extraction device

The inclusion of octadecyl groups using trimethoxy(octadecyl)silane can be performed by sol-gel or grafting methods. The sol-gel reaction, which is usually performed in ethanol, produces an efficient coating with a crystalline conformation. The grafting approach is performed in toluene and its results in a lower coverage. However, this coating presents a liquid-like chain conformation [23] which may enhance the interaction and stabilization of a given solvent on the disk surface. Therefore, the latter approach was selected. The functionalization of the borosilicate disk consists of several and well defined steps. First of all, the borosilicate disk was activated by its immersion in a sulfuric acid: hydrogen peroxide (2:1 v/v) solution for 20 min at 100 ºC. The activated disk was washed with Milli-Q water up to neutral pH and dried in an oven at 80 ºC for 12 h. In a second step, the activated disk was introduced in a 50 mL of dry toluene containing 0.5 mL of trimethoxy(octadecyl)silane, the mixture being refluxed during 12 h at 120 ºC. The obtained disk was finally washed several times with ethanol to remove toluene and unreacted products and dried in an oven at 80 ºC for 2 h. The derivatized disk was characterized by FTIR (**Figure S1**) and by a colorimetric assay with phenolphthalein (**Figure S2**). Both assays are described in the supplementary information section.

The synthesized disk was adapted to a section of a 20-mL plastic syringe (20 mm internal diameter and 15 mm in height) as it is shown in **Figure 1A**. Previously, the section was pierced to create windows that favor the mass transference [6]. Moreover, the unit is pierced by a cylindrical magnetic rod protected from oxidation with a thin layer of polyvinyl chloride, which allows the stirring of the solution at high rates. Before the extraction, the octadecyl-modified disk should be impregnated with an appropriate solvent (as it is shown in **Figure 1B**) to form a supported liquid solvent which is the responsible for the analytes extraction.

### 2.4. Extraction procedure

The proposed extraction procedure is based on the principles of liquid phase microextraction. In this case, the $C_{18}$-modified borosilicate disk is inserted into the unit and impregnated with 300 µL of toluene. Then, the extraction unit is introduced into a 1-L graduated cylinder containing 500 mL of aqueous standard or sample with the ionic strength adjusted to a final concentration of sodium chloride of 50 g/L. Later on, the extraction unit is stirred at 1400 rpm for 30 min in a magnetic stirrer (Velp Scientifica, Milan, Italy) to perform the extraction of the analytes. After the extraction, the $C_{18}$-modified disk is withdrawn and directly inserted on the lower section of a 20-mL syringe (**Figure 1C**). 1 mL of methanol is used to sweep along from the $C_{18}$-modified borosilicate disk the remaining toluene containing the triazine herbicides. This process is performed in a SPE vacuum manifold (Supelco, Bellefonte, PA).

To improve the sensitivity, the extract is evaporated to dryness under a nitrogen stream. This step is possible since no losses by evaporation are observed for the target analytes. The residue is finally resuspended in 100 µL of methanol and placed on a Total Recovery ® vial (Waters Corp.) for UPLC analysis. Between extractions, the disk is rinsed with water and methanol to avoid potential carry-over effects.

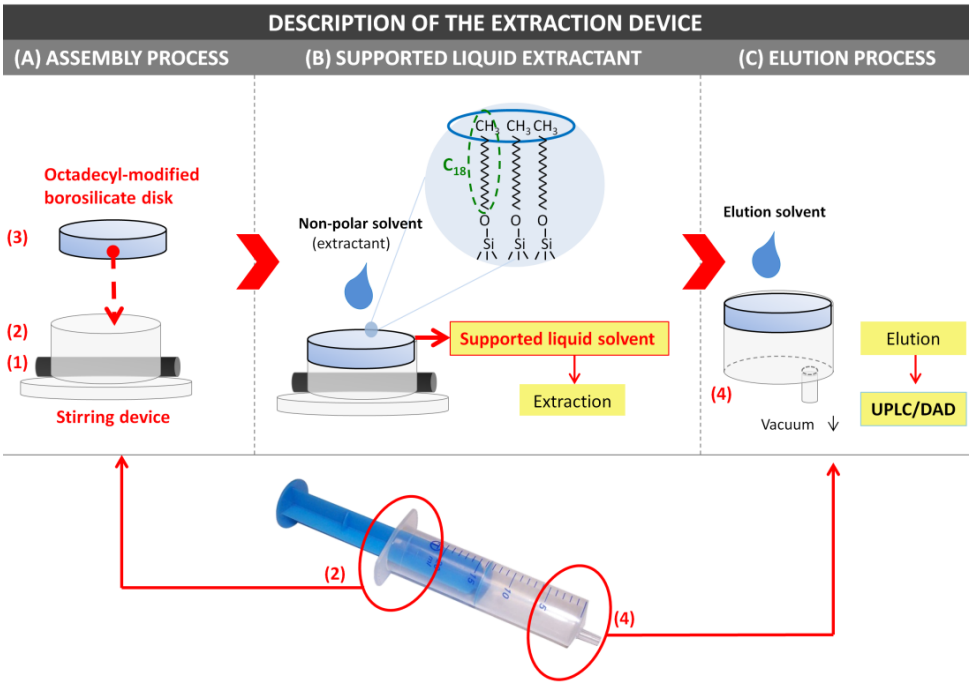


**Figure 1.** Description of the extraction device. (A) The assembly process requires: (1) cylindrical magnet rod; (2) section of a 20-mL plastic syringe and (3) octadecyl-modified borosilicate disk. (B) The supported liquid solvent is formed by hydrophobic interactions between the solvent and the disk support. (C) The elution process requires a (4) section of a 20-mL plastic syringe. For details, see text.

## 3. RESULTS AND DISCUSSION

### 3.1. Optimization of the extraction conditions

Different variables, which are summarized in **Table 1**, may affect the efficiency of the extraction procedure and therefore their effects on the analytes extraction were considered in depth in the optimization process. **Table 1** also reflects their initial values, the interval studied and the optimum values. The optimization was performed under a univariate approach although two couples of variables were considered jointly since they are directly

related. An aqueous standard solution containing the nine triazines at a concentration of 100 µg/L was used in these studies.

**Table 1**. List of the variables involved in the extraction process.

| Variable | Initial value | Interval studied | Optimum value |
|---|---|---|---|
| Extraction solvent and | | Hexane, chloroform, ethyl acetate, toluene, methanol | Toluene |
| type of disk | | Bare and $C_{18}$ borosilicate disks | $C_{18}$ borosilicate disk |
| Ionic strength (g/L NaCl) | 0 | 0 - 200 | 50 |
| Stirring rate (rpm) | 900 | 0 - 1400 | 1400 |
| Sample volume (mL) | 200 | 200 - 750 | 500 |
| Extraction time (min) | 10 | 5 - 60 | 30 |
| Elution volume (mL) | 1 | 0.5 - 1.5 | 1 |
| Volume for redissolution (µL) | 500 | 100, 500 | 100 |

### *3.1.1. Selection of the extraction solvent and the type of borosilicate disk*

The appropriate selection of the solvent in a liquid phase microextraction technique is a critical step. The solvent should fulfill some requirements being the affinity towards the target analytes the most relevant. However, other features can be significant from the operational point of view.

In this approach, the final extract is evaporated to achieve a sensitivity enhancement and so the solvent should present an appropriate volatility. Moreover, the hydrodynamic stability of the solvent in the support, the borosilicate disk, is essential to allow its recovery after the extraction. Both variables, the type of solvent and the type of borosilicate disk, were evaluated jointly. For this purpose, different solvents (hexane, ethyl acetate, chloroform,

toluene and methanol) and two borosilicate disks (bare and modified with octadecyl groups) were considered.

The results, expressed in terms of enrichment factors (EFs), are presented in **Figure 2**. Attending to the results, different conclusions can be inferred. First of all, the supported solvent is the responsible for the extraction of the target analytes since very low EFs are obtained in its absence. Secondly, the $C_{18}$-functionalized disk provides better EFs than the bare disk and this fact can be ascribed to the hydrodynamic stability, through hydrophobic interactions of the solvent in the derivatized disk. Finally, as expected from the extraction basis, the polarity of the analytes and the extractant is the driving force of their interaction. In this sense, toluene provides the best EFs for all the analytes although for simazine, the most polar analyte, chloroform is also an alternative. However, the use of chloroform resulted in lower precision values since its interaction with the hydrophobic disk is less marked. In the same way, hexane can be an alternative for the more hydrophobic compounds.

Special attention should be paid to the role that methanol may play in this type of extraction. Although it is miscible with water, its use drives to good enrichment factors for the hydrophobic analytes. Methanol acts as conditioning agent of the octadecyl groups which extract the hydrophobic analytes following the solid phase extraction principles.

Attending to the results, the combination of $C_{18}$-disk and toluene was considered for further studies.

The high porosity of the borosilicate disk is advantageous since it increases the surface contact between the extractant solvent and the sample. However, the porosity involves the use of high solvent amounts, especially if they are compared with classical microextraction techniques, in order to cover efficiently the disk pores.

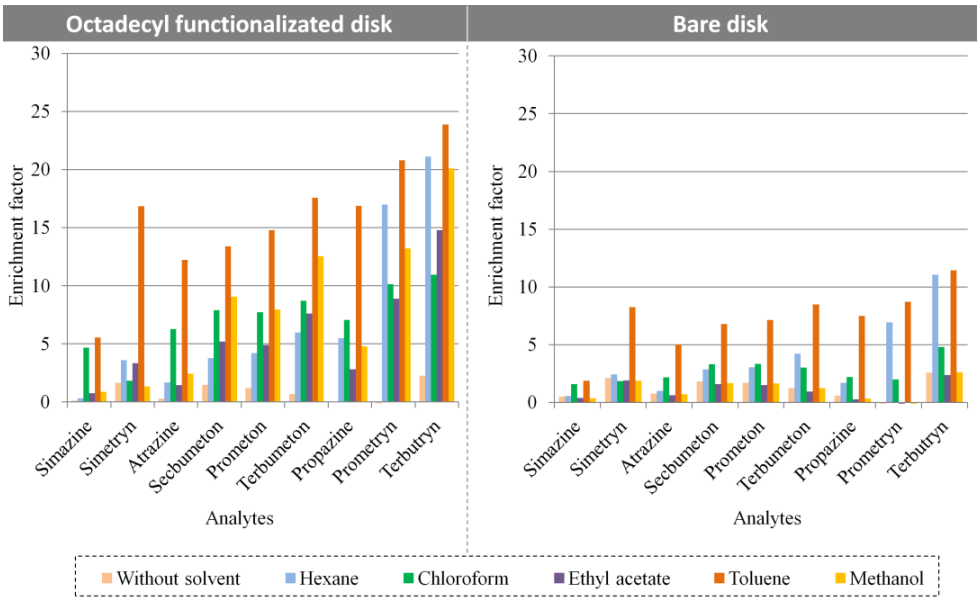


**Figure 2.** Effect of the type of disk and the organic solvent in the extraction of the target analytes. For details, see text.

### *3.1.2. Effect of the ionic strength on the extraction*

Ionic strength may play a key role in microextraction techniques. In fact, it may present two contradictory effects. On the one hand, it can decrease the solubility of the target analytes in water, by the so-called salting-out effect, favoring its transference to the supported liquid solvent. This effect is especially interesting to enhance the extraction of analytes with intermediate polarity. On the other hand, ionic strength may increase the viscosity of the sample, negatively affecting the Nernst layer and the extraction kinetics. This aspect affects in the same extension to all analytes regardless of their polarity.

The influence of the ionic strength was evaluated using sodium chloride (NaCl) as model electrolyte in the range of 0 to 200 g/L. The extraction of all the analytes is positively

affected at lower ionic strength (showing a prevalence of the salting out effect) whereas at higher concentration (over 50 g/L) a decrease in the EFs was observed (due to the second effect). However the extraction of simazine, the less hydrophobic analyte, presents an increase in the EFs with the ionic strength at low concentration of NaCl, remaining almost constant at higher concentration values. Attending to the results, 50 g/L of sodium chloride was selected as optimum value for further studies.

### *3.1.3. Effect of the stirring rate and sample volume on the extraction*

According to our previous studies about stirring extraction devices, the sample volume and the stirring rate are related variables since both define the distance between the solution vortex and the extraction element. Overall, when higher samples volumes are processed, higher stirring rates can be used. For this reason, both variables were optimized together considering three different sample volumes (200, 500 and 750 mL) and five different stirring rates (0, 500, 900, 1100 and 1400 rpm). The optimization process was performed taking into account all the target analytes, but for simplicity, **Figure 3** shows the bivariant effect on the extraction (EFs) of prometryn by means of a contour surface graph. The results obtained for the rest of the analytes are presented in the *Supplementary Material* (**Figures S3, S4, S5, S6, S7, S8, S9** and **S10**). Three different behaviors were observed depending on the sample volume. For 200 mL, the EFs increased with the stirring rate up to 1100 rpm. For higher velocities, a vortex was created directly above the extraction device reducing the contact between the sample and the extractant. When 500 mL of sample volume was employed, the EFs increased almost linearly with the stirring rate. For 750 mL, the EFs increase up to 1100 rpm, remaining constant for higher velocities. According to the obtained results, a sample volume of 500 mL and a stirring rate of 1400 rpm were chosen as the optimal values to carry out the extraction procedure.

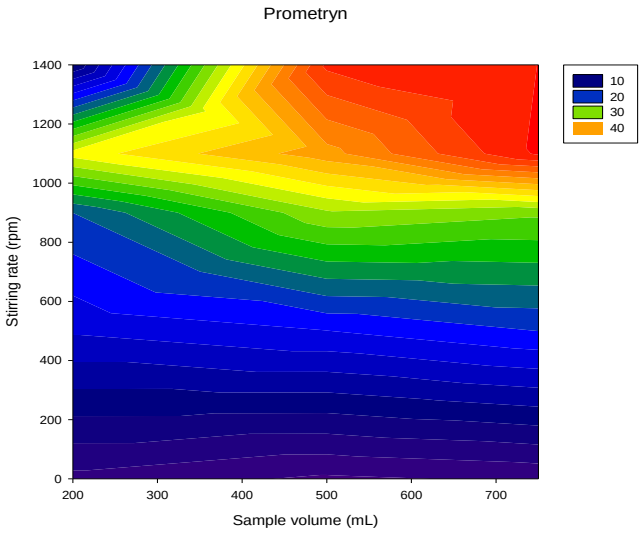


**Figure 3.** Bivariant effect of sample volume and stirring rate on the enrichment factors for prometryn. For details, see text. The data obtained for the rest of analytes are available in *Supplementary Material*.

### *3.1.4. Effect of the extraction time*

The influence of the extraction time or stirring time is also an important variable in the extraction process. This variable was investigated in the range from 5 to 60 min and the results, which are depicted in **Figure 4**, showed two different behaviors.

The extraction of the most hydrophobic analytes (PMT and TBT) increases markedly and almost linearly with the time up to 30 min, while from 30 to 60 min the increase was less pronounced. On the other hand, the extraction of analytes with intermediate polarity slightly increases up to 30 min, remaining almost constant for higher times. Furthermore, the reproducibility became worse with the extraction time and this fact can be ascribed to the instability (potential detachment or solubilization) of the extractant when longer

extraction times are employed. Therefore, 30 min was fixed as the optimum value as a compromise between the sample throughput and sensitivity.

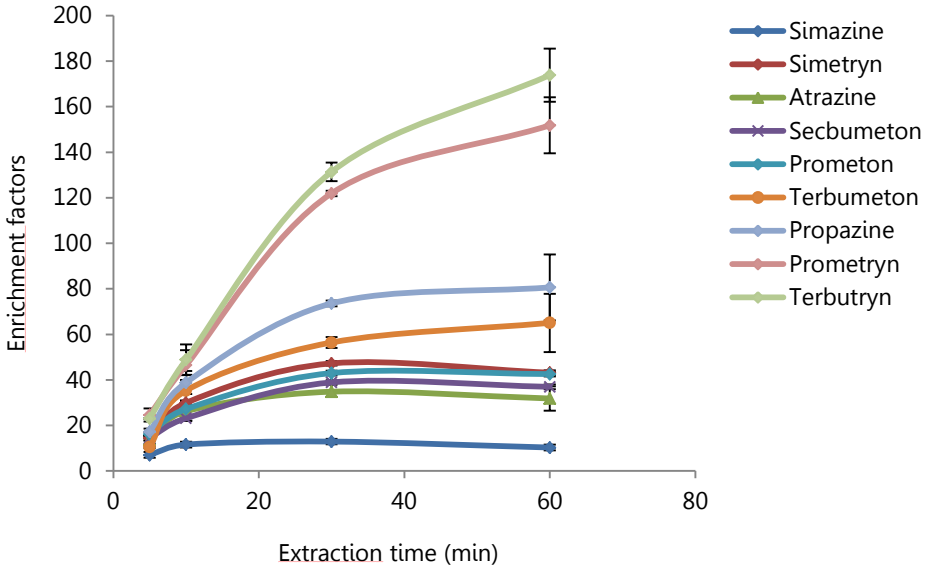


**Figure 4.** Effect of the extraction time on the enrichment factors of the analytes.

### *3.1.5. Elution process*

Once the analytes have been conveniently extracted, they should be eluted for the subsequent chromatographic analysis. For this purpose, the $C_{18}$-modified disk containing the toluene with the retained analytes is placed into a 20-mL syringe section, which is located in a SPE vacuum manifold. The elution is performed passing the solvent through the disk. Methanol was selected as eluent according to the literature [24] as it provides the best elution results not only in terms of absolute recovery, but also in precision.

The elution volume was studied taking into account that the final extract will be evaporated under a $N_2$ stream not only for sensitivity enhancement but also in order to allow the

chromatographic separation since the majority of the evaluated solvents were immiscible with the mobile phase. Therefore, the volume should be higher enough to provide an efficient elution of the analytes but lower enough to allow a rapid evaporation. The elution volume was studied in the range from 0.5 to 1.5 mL (data not shown), 1 mL of methanol being selected as a compromise between both effects. Finally, the resuspended volume was also evaluated (100 µL and 500 µL) and as was be expected higher EFs were obtained for 100 µL.

### 3.2. Analytical figures of merit

The analytical figures of merit of the proposed method are summarized in **Table 2.** The calibration curves for the analytes were constructed by using eight working aqueous standards prepared at controlled concentrations which were subjected to the optimized extraction procedure. The method was characterized on the basis of its linearity, sensitivity, precision and recovery.

The sensitivity of the method was evaluated according to the limit of detection (LOD). LODs, calculated using a signal-to-noise ratio (S/N) of 3, ranged from 0.14 µg/L (atrazine) to 0.56 µg/L (terbumeton). Limits of quantification, calculated using a S/N of 10, varied between 0.47 µg/L (atrazine) to 1.84 µg/L (terbumeton). The linearity was kept until 100 µg/L for all analytes. The precision was evaluated under repeatability conditions at two different concentration levels: 5 µg/L and a concentration closer to the limit of quantification of each analyte. As it can be seen in **Table 2,** the repeatability (expressed as relative standard deviation, RSD) ranged from 0.8% to 7.3% and from 2.8% to 10.7% at 5 µg/L and LOQ, respectively. Moreover, **Table 2** also summarizes the absolute extraction recovery (AER) values obtained with the proposed extraction procedure. These values ranged from 1.6% (simazine) to 16.8% (terbutryn) and they are in agreement with those obtained in other microextraction techniques, since these procedures are non-exhaustive extraction methods [26].

**Table 2.** Analytical figures of merit of the proposed method for the determination of triazine herbicides in water.

| Analyte | LOD[a] (µg/L) | LOQ[b] (µg/L) | Precision[c] (%) | | EFs[d] | AER[e] (%) |
|---|---|---|---|---|---|---|
| | | | At 5 µg/L (n=3) | At LOQ[b] (n=3) | | |
| Simazine | 0.17 | 0.56 | 4.9 | 9.6 | 79 | 1.6 |
| Simetryn | 0.18 | 0.61 | 5.0 | 8.8 | 387 | 7.8 |
| Atrazine | 0.14 | 0.47 | 4.7 | 7.2 | 271 | 5.4 |
| Secbumeton | 0.25 | 0.83 | 1.8 | 3.9 | 291 | 5.8 |
| Prometon | 0.20 | 0.66 | 6.1 | 8.5 | 323 | 6.5 |
| Terbumeton | 0.56 | 1.84 | 0.8 | 2.8 | 419 | 8.4 |
| Propazine | 0.22 | 0.73 | 1.7 | 8.0 | 485 | 9.7 |
| Prometryn | 0.22 | 0.73 | 0.8 | 10.7 | 782 | 15.7 |
| Terbutryn | 0.18 | 0.59 | 7.3 | 8.5 | 839 | 16.8 |

[a] *LOD,* limit of detection, calculated for a S/N of 3.
[b] *LOQ,* limit of quantification, calculated for a S/N of 10.
[c] Precision expressed as relative standard deviation.
[d] EFs, enrichment factors. The theoretical maximum value is 5000.
[e] *AER,* absolute extraction recovery.

Finally, **Table 3** compares the proposed method with other methodologies for the resolution of the same analytical problem [27-38]. These approaches cover a wide range of extraction as well as instrumental techniques. For simplicity, the discussion has been focused on liquid phase microextraction approaches and techniques that integrate stirring and extraction in the same device. According to the results, the new proposal is the one that allows processing the larger sample volumes and the second that provides the highest enrichment factors. Furthermore, precision levels are comparable with the other approaches.

The sensitivity could be considered the weak factor in the comparison with other methodologies that provide lower detection limit. This fact can be ascribed to two different reasons. The first one is related to the instrumental technique used for analytes

determination. On the other hand, in some cases the extractant volume is quite similar to the volume which is finally introduced in the instrument, which has a clear influence of the sensitivity of the determination.

**Table 3.** Relative recovery study performed on real samples spiked with the analytes at a concentration of 3 µg/L .

| Analyte | Brook water samples (R% ± SD) | | |
|---|---|---|---|
| | I | II | III |
| Simazine | 77 ± 9 | 82 ± 10 | 89 ± 10 |
| Simetryn | 106 ± 9 | 97 ± 9 | 101 ± 9 |
| Atrazine | 84 ± 7 | 97 ± 7 | 95 ± 7 |
| Secbumeton | 107 ± 4 | 95 ± 4 | 103 ± 4 |
| Prometon | 99 ± 9 | 110 ± 8 | 120 ±8 |
| Terbumeton | 84 ± 3 | 83 ± 3 | 90 ± 3 |
| Propazine | 70 ± 8 | 80 ± 8 | 80 ± 8 |
| Prometryn | 72 ± 10 | 74 ± 11 | 72 ± 11 |
| Terbutryn | 79 ± 9 | 77 ± 8 | 78 ± 8 |

*R%* extraction recovery, *SD* standard deviation (n=3).

### 3.3. Analysis of water samples

Once optimized and analytically characterized, the proposed method was applied for the determination of the target triazines in water samples. A recovery study was performed in order to evaluate the applicability of the proposed method to determine triazine herbicides in brook waters. First of all, the samples were analyzed in order to find any potential presence of the analytes and later on they were spiked at 3 µg/L concentration level. From the results listed in **Table 4**, it can be concluded that the extraction process fulfills the 70-130% recovery criterion [39]. The results show the potential of the proposed stir $C_{18}$-modified borosilicate disk for the extraction of the nine triazine herbicides from natural environmental waters.

**Table 4.** Comparison of the proposed extraction procedure with other published methods for the extraction of triazine herbicides from waters.

[a]*Acronyms: SLM,* supported liquid membrane; *HF-LPME,* hollow fiber liquid phase microextraction; *SDME,* single drop microextraction; *LLSME,* liquid-liquid-solid microextraction; *DLLME,* dispersive liquid-liquid microextraction; *IL-DLPME,* ionic liquid dispersive liquid phase microextraction; *DLLME-SFO,* dispersive liquid-liquid microextraction based on solidification of floating organic drop; *VSLLME,* vortex-assisted surfactant-enhanced-emulsification liquid-liquid microextraction; *PU,* polyurethanes; *SBSE,* stir bar sorptive extraction.

| Extraction technique[a] | Sample volume (mL) | Extractant | Instrumental technique[b] | Extraction time (min) | LOD[c] (µg/L) | Theoretical maximum EFs[d] | EFs[d] | Relative recovery (%) | Precision (% RSD[e]) | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| HF-LPME | 3 | Toluene (3µL) | GC-MS | 20 | 0.007-0.063 | 1000 | 42-208 | 90-112 | 0.9-3.4 | [27] |
| HF-LPME | 250 | *TOPO (10%) and TBP (10%) in DHE (26 µL) | LC-MS/MS | 120 | 0.026-0.061 | 9615 | 5-2000 | n.e | 0.2-5.8 | [28] |
| SDME | 1 | Butyl acetate (3 µL) | GC-MS | 15 | 0.02-0.4 | 333 | 38-189 | 18-90 | 5.6-10.9 | [29] |
| SDME | 5 | 1-Octanol (20 µL) | HPLC-UV | 40 | 0.03-0.04 | 1000 | 76-91 | 88-110 | 5.0-6.7 | [30] |
| LLSME | 3 | Toluene (6 µL) | HPLC-UV | 30 | 0.012-0.02 | 500 | 86-147 | 17-29 | 1.2-9.6 | [31] |
| DLLME | 5 | Chlorobenzene (12 µL) | GC-MS | 3 | 0.021-0.12 | 2500 | 151-722 | 85-113 | 2.4-7.2 | [32] |
| IL-DLPME | 10 | Ionic liquid (60 µL) | HPLC-DAD | 30 | 0.46-0.89 | 500 | 270-384 | 50-85 | 7.9-9.8 | [33] |
| DLLME | 5 | Chloroform (100 µL) | HPLC-DAD | 10 seconds | 0.05-0.1 | 333 | 183-221 | 84-102 | 2.9-5.2 | [34] |
| DLLME-SFO | 5 | Undecanol (10µL) | GC-MS | 3 | 0.008-0.033 | 500 | 195-322 | 100-117 | 0.03-5.1 | [35] |
| VSLLME | 5 | Chloroform (100 µL) | CE-UV/Vis (DAD) | 3 | 0.41-0.55 | 500 | 265-310 | 81-107 | 3.9-4.6 | [36] |
| SBSE (PU) | 25 | (71 µL) | HPLC-DAD | 360 | 0.1-0.5 | 352 | n.e | 20-62 | 4.3-6.7 | [37] |
| SBSE | 50 | PDMS (1mm) | LC-MS/MS | 60 | 0.03-1 | 50 | n.e | 15-31 | 7-12 | [38] |
| Stir $C_{18}$-modified borosilicate disk (LPME) | 500 | Toluene (300 µL) | UPLC-DAD | 30 | 0.14-0.56 | 5000 | 79-839 | 70-120 | 0.8-7.3 | This method |

[b]*Acronyms: HPLC,* high performance liquid chromatography; *UV,* ultraviolet; *GC,* gas chromatography; *MS,* mass spectrometry; *DAD,* diode array detection; *CE,* capillary electrophoresis; *UPLC,* ultra performance liquid chromatography.
[c]*LOD,* limit of detection.
[d]EF, enrichment factor.
[e]*RSD,* relative standard deviation.
*TOPO (tri-n-octylphosphine oxide), TBP (tri-n-butyl phosphate), DHE (di-n-hexylether).

## 4. CONCLUSIONS

In the present article, a novel technique which combines the liquid phase microextraction and the well-known advantageous effect of stirring in the same device is presented.

The main advantages and disadvantages of the proposal have been established following a SWOT (strengths, weakness, opportunities and threats) analysis, which is summarized in **Figure 5**.

The new extraction device requires commercial elements and simple apparatus to be performed. Moreover, it allows analyzing large sample volumes (up to 500 mL) which involves high potential enrichment factors (the practical ones obtained for the model analytes ranged from 79 to 839), in a reasonable extraction time. These high EFs enhance the sensitivity of the developed methodology providing detection limits in the low microgram per liter range. The extraction unit is reusable since no carry-over between samples was observed and the use of a protected magnetic rod avoids the appearance of oxide and the inherent degradation of the unit.

The proposed method fulfills, except for terbumeton, the sensitivity requirements established by US-EPA and also the EU requirements which establish a limit of 1 μg/L for individual herbicides and 3 μg/L for total herbicides in surface waters. An additional improvement in sensitivity is desirable in order to achieve the more restrictive limit imposed by EU for drinking waters. This could be enhanced using a more sensitive instrumentation (e.g MS) or using different sample/eluent ratios.

Borosilicate disks are useful extraction materials because of their good sample-to-adsorbent contact surface due to their high porosity. Moreover, the potential chemical modification makes them very versatile tools since different extraction groups can be introduced. In this sense, the extraction device can work under the liquid phase or solid phase microextraction formats (see extraction solvent optimization section).

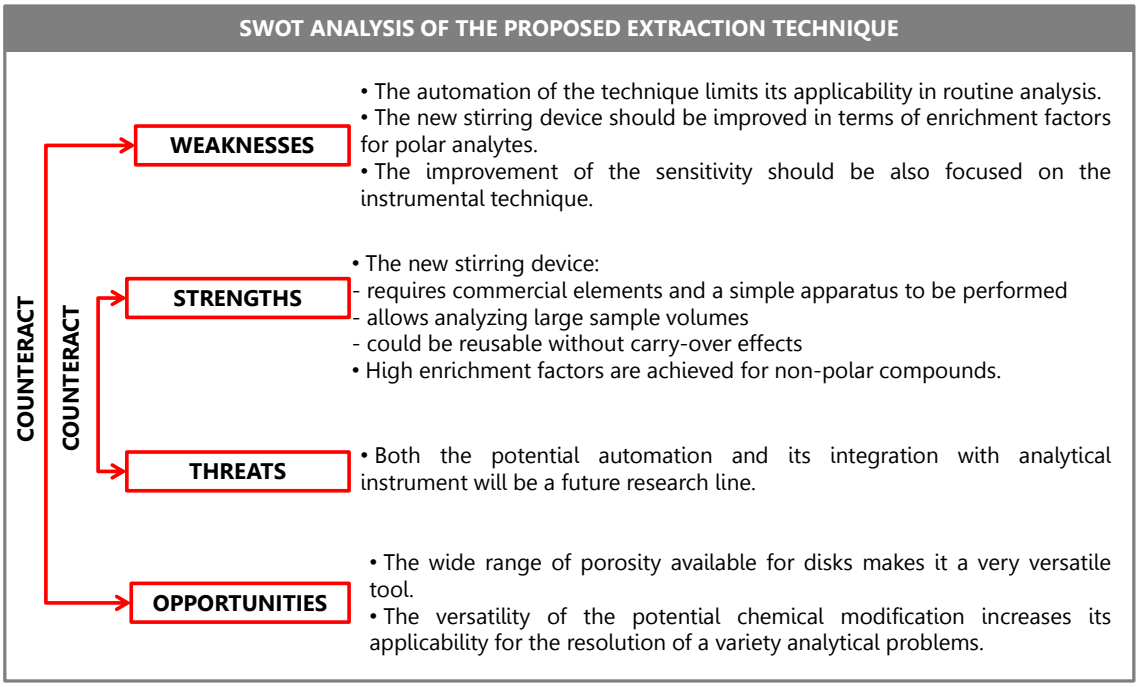


**Figure 5**. SWOT analysis of the proposed extraction technique. Strengths and weaknesses are obtained after a critical evaluation of the proposal. Opportunities and threats are obtained taking into consideration the main requirements for sample treatment technique.

Being critical, some aspects have to be improved. The weakness of the technique lies on the lack of automation which may limit its applicability in routine analysis. Moreover, the new proposal requires higher sample and extractant volumes than other liquid phase microextraction techniques. The requirement of large sample volumes depends directly on the real problem and the analytical instrumentation. If high enrichment factors are required to solve a given analytical problem, high sample volumes should be processed. However, the sample volume could be reduced if the target sensitivity is lower. On the other hand, the extractant volume is fixed by the porosity and dimension of the disks and therefore the use of less porous or smaller supports may allow the use of lower extractant volumes.

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## SUPPLEMENTARY MATERIAL

### Experimental section: octadecyl-modified borosilicate disk characterization.

The synthesized octadecyl disks were characterized by FT-IR spectroscopy and colorimetric assay. Infrared measurements were performed in a Bruker Tensor37 FT-IR spectrometer, equipped with a diamond ATR cell with a circular surface of 3 mm diameter and three internal reflections. A Deuterated Triglycine Sulfate (DTGS) detector was used for spectra acquisition. Spectra were collected between 4000 and 600 $cm^{-1}$ at a 4 $cm^{-1}$ resolution with 128 coadded scans each. Data collection was made using OPUS software (Bruker, Ettligen, Germany).

The IR spectrum obtained for the octadecyl-modified disk, which is presented in **Figure S1**, clearly shows the absorption bands of the borosilicate support. Among them, the bands observed at 906 $cm^{-1}$ (B–O bending vibration), 1030 $cm^{-1}$ (Si-O-Si asymmetric stretching vibration) and 1379 $cm^{-1}$ (B-O stretching vibration) can be highlighted. Moreover, two additional bands of lower intensity can be observed in the 2900-300 $cm^{-1}$. These bands can be endorsed to the C-H stretching vibrations of the octadecyl phase.

The low intensity of the latter can be ascribed to the high porosity of the disk which makes difficult the acquisition of the spectrum under an ART technique. In addition, this low intensity explains a crucial aspect in this application. The $C_{18}$ coverage is enough to achieve the immobilization of the organic solvent on the disk but it is not high enough to extract by its self in a large extent. Therefore, the coverage of the disk should be improved for its use under a classical SPE format.

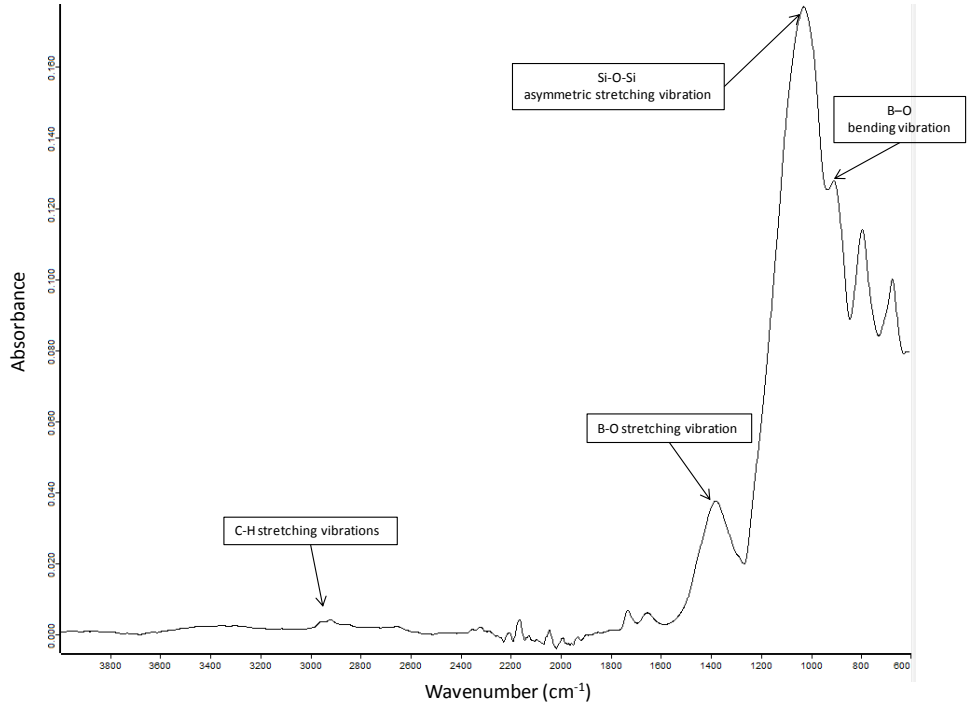


**Figure S1.** Infrared spectrum of the octadecyl-modified borosilicate disk.

Finally, a colorimetric assay using phenolphthalein as indicator was carried out. Both borosilicate disks (bare and modified with octadecyl groups), were conditioning with methanol. In a second step, each disk was introduced in 75 mL of Milli Q water (pH 3) containing 0.5 mL phenolphthalein and stirred at 900 rpm for 10 min. Later on, the mixture was discarded and the disks were washed with Milli Q water at pH 3 four times in order to remove physisorbed phenolphthalein. Finally, 75 mL of Milli Q water at pH 11 were added to each disk and stirred 5 min at 900 rpm. As it is shown in **Figure S2**, only octadecyl-

modified disk developed a red color as a consequence of the prior retention of phenolphthalein by hydrophobic interactions.

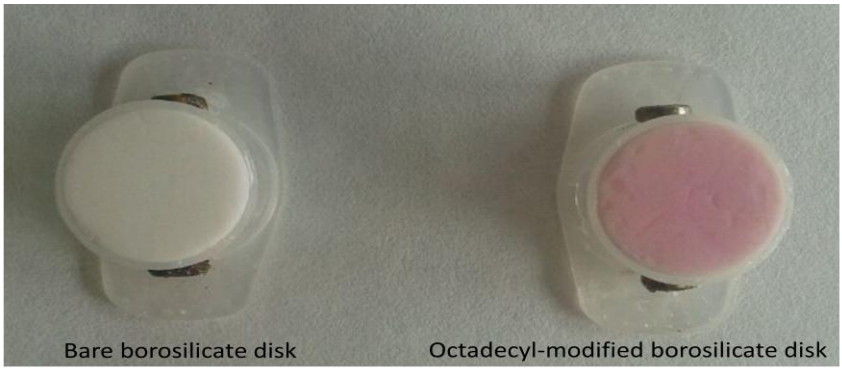


**Figure S2**. Characterization of the synthesized by colorimetric assay with phenolphthalein.

**Results and discussion: Bivariant effect of sample volume and stirring rate on the enrichment factors for the target analytes.**

For brevity, the manuscript only presents the data obtained for prometryne which is selected as representative analyte. Although all the analytes presented similar behaviors, the optimum conditions are slightly different for all of them. In the following figures, the data obtained for all the analytes assayed are presented.

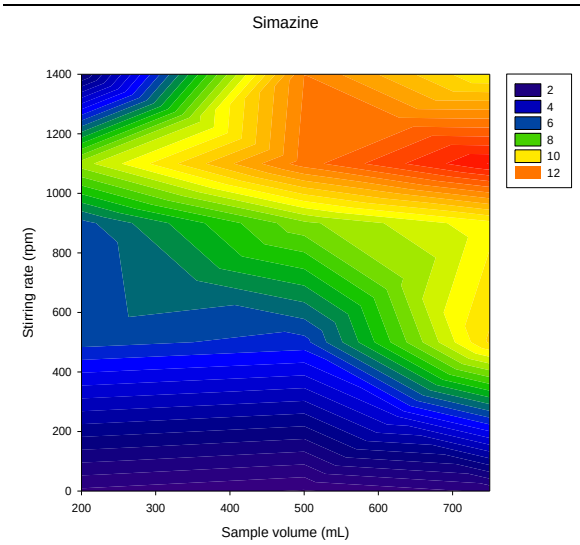


**Figure S3**. Bivariant effect of the sample volume and stirring rate on the analytical signal obtained for a standard solution containing simazine at 100 µg/L.

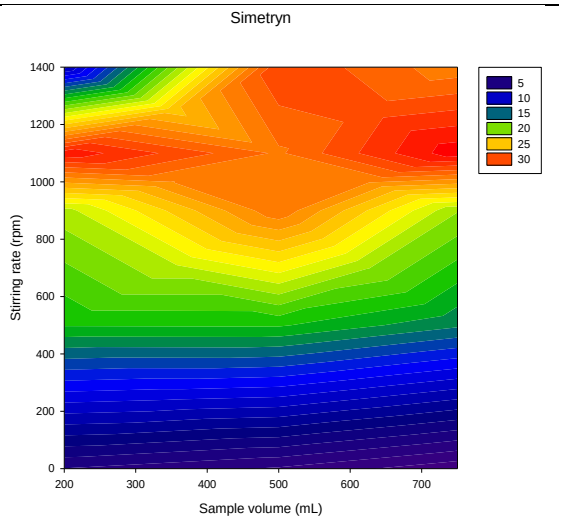


**Figure S4.** Bivariant effect of the sample volume and stirring rate on the analytical signal obtained for a standard solution containing simetryn at 100 µg/L.

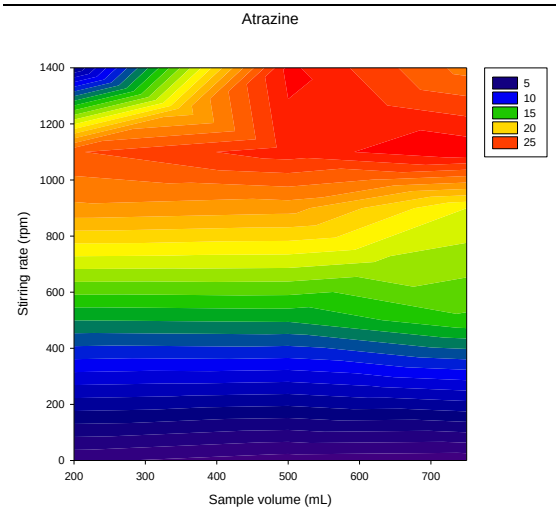


**Figure S5**. Bivariant effect of the sample volume and stirring rate on the analytical signal obtained for a standard solution containing atrazine at 100 µg/L.

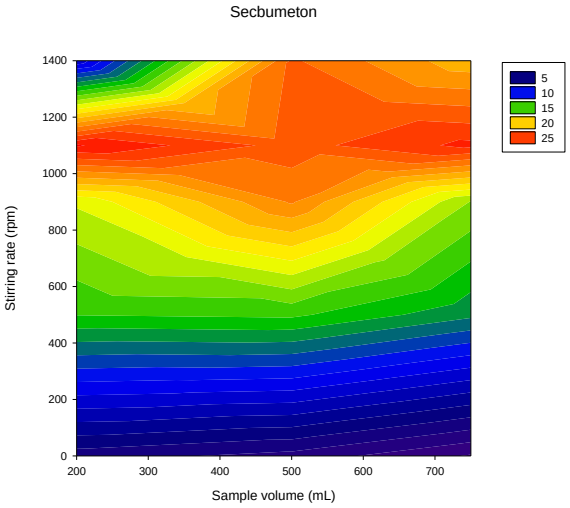


**Figure S6**. Bivariant effect of the sample volume and stirring rate on the analytical signal obtained for a standard solution containing secbumeton at 100 µg/L.

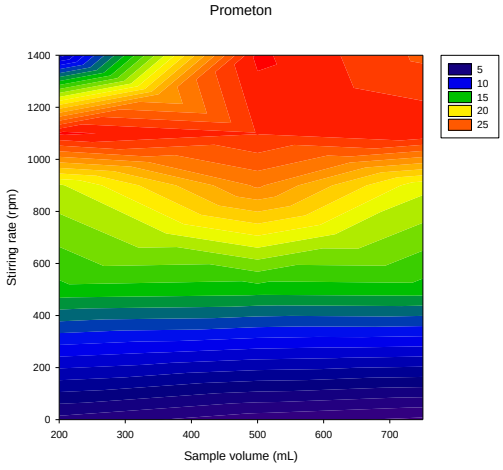


**Figure S7**. Bivariant effect of the sample volume and stirring rate on the analytical signal obtained for a standard solution containing prometon at 100 µg/L.

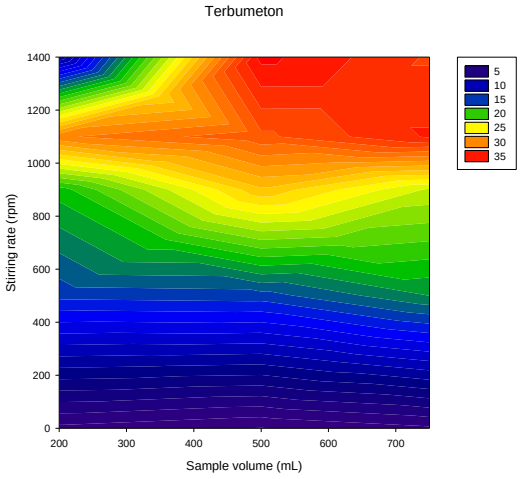


**Figure S8.** Bivariant effect the sample volume and stirring rate on the analytical signal obtained for a standard solution containing terbumeton at 100 µg/L.

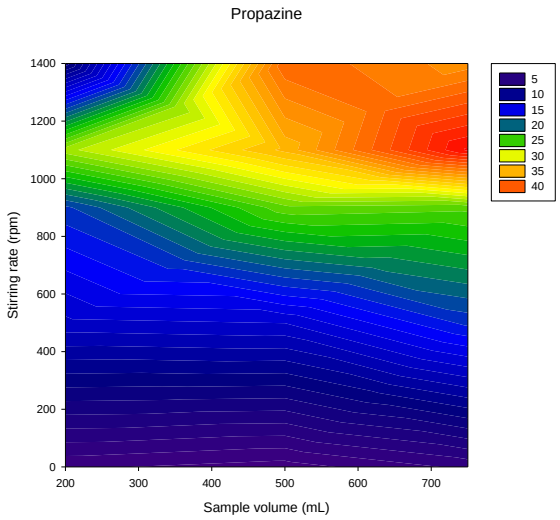


**Figure S9**. Bivariant effect of the sample volume and stirring rate on the analytical signal obtained for a standard solution containing propazine at 100 µg/L.

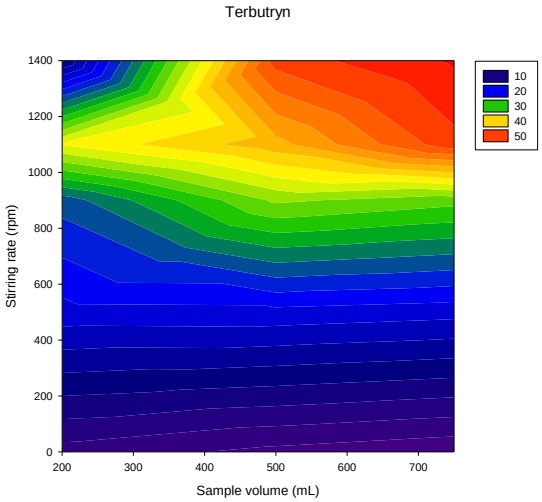


**Figure S10**. Bivariant effect of the sample volume and stirring rate on the analytical signal obtained for a standard solution containing terbutryn at 100 µg/L.

CAPÍTULO 7

# Stir membrane liquid microextraction for the determination of paracetamol in human saliva samples

*Bioanalysis 5 (2013) 307-315*

FUTURESCIENCE

**Bioanalysis**

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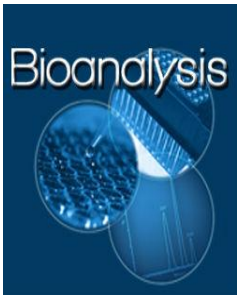


# Stir membrane liquid microextraction for the determination of paracetamol in human saliva samples

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In this article, stir membrane liquid microextraction is adapted for the analysis of volume-limited biological samples, using as model analytical problem the determination of paracetamol in human saliva by liquid chromatography (LC) with ultraviolet detection (UV). A three-phase microextraction mode is used for the extraction of the target analyte taking advantage of its acid-base properties. All the variables involved in the extraction have been studied and optimized in depth. The method has been analytically characterized on the basis of its linearity, sensitivity and precision. The limit of detection is 0.5 µg/L while the repeatability, expressed as relative standard deviation (RSD), is better than 14.2%. A 10-fold preconcentration was obtained which involves an absolute recovery value of 25%. Moreover, the relative recovery is very close to the 100%. Finally, the proposed method has been used to perform a pharmacokinetic study of paracetamol.

***Keywords:*** *Stir membrane liquid-liquid-liquid microextraction; Volume-limited biological samples; Saliva; Paracetamol.*

## 1. INTRODUCTION

Although direct analysis of biological samples is possible in some cases [1], in most of the situations, a previous sample pretreatment is required due to the complex biological matrices and/or the low concentration of the target analytes. In this context, sample pretreatment techniques play a crucial role in the bioanalytical field since they allow the isolation and the preconcentration of the target compounds improving the selectivity and sensitivity of the analytical methodologies, respectively [2].

Classic liquid-liquid extraction (LLE) [3] and solid phase extraction (SPE) [4] have been widely used in bioanalysis due to their good performance. Both techniques are especially useful for processing large sample volumes since they provide low preconcentration factors when they are applied to low sample volumes. However, some biological specimens present a volume limitation and therefore new extraction procedures than the classic ones are required in order to isolate and preconcentrate target analytes from such samples. Microextraction techniques [5], both in the solid phase (SPME) [6, 7] and liquid phase (LPME) [8] formats have been extensively employed in bioanalysis and they provide high enrichment factors even when low sample volumes, in the µL-mL range, are processed.

Among the volume-limited biological specimens, saliva is quite interesting due to its non-invasive, rapid, economical and easy sampling. Moreover, for many drugs and biomolecules an excellent correlation between saliva and blood concentrations has been observed [9]. This fact opens the possibility of using saliva analysis to obtain bioanalytical information. In this context, SPME has been used for the isolation of biologically important compounds from saliva under different extraction modes such as stir bar sorptive extraction [10], and in-tube SPME [11]. Micro solid phase extraction (µ-SPE) in the form of microextraction in packed sorbent (MEPS) [12] has been also employed in this context. In the same way, LPME has been successfully used as pretreatment technique in saliva analysis [13, 14].

Membrane-based liquid phase microextraction has attracted much attention in bioanalysis due to its miniaturized nature, simplicity and efficiency. Moreover, the polymeric membrane provides a selectivity enhancement by sieving, avoiding the presence of high size biomolecules in the final extracts. Hollow fiber protected liquid phase microextraction (HF-LPME) [15, 16] and electrokinetic membrane extraction (EME) [17], both developed by Pedersen-Bjergaard *et al.*, are clear paradigms of these techniques.

Recently, stir membrane extraction has been proposed as a novel technique that integrates in the same device the extraction and stirring elements [18]. The extraction technique has been also applied successfully in the LPME context both in the two-phase [19] and three-phase modes [20]. Although its usefulness in bioanalysis has been recently demonstrated by the extraction of non-steroidal anti-inflammatory drugs from urine samples [21], its application to volume-limited biological specimens has not been yet considered since it requires an adaptation of the extraction device.

In the present paper, we report the adaptation of the stir membrane liquid phase microextraction for the analysis of volume-limited biological samples. The extraction device is modified to reduce the sample volume to the low mL range which also allows the easy stirring of the extraction unit. The new proposal has been evaluated using the determination of paracetamol in saliva samples as model analytical problem. Paracetamol or acetaminophen is worldwide employed thanks to its analgesic and antipyretic properties [22]. According to its pharmacokinetics, it is mainly transferred into the saliva by passive diffusion [23]. Numerous studies have reported the agreement between paracetamol saliva concentration with those found in serum or plasma [24, 25]. The intention of the article is to evaluate the potential of the microextraction technique, which can be easily adapted to other bioanalytical processes.

## 2. EXPERIMENTAL SECTION

### 2.1. Reagents and materials

All the reagents were of analytical grade or better. Paracetamol was supplied by Sigma–Aldrich (Madrid, Spain). A stock standard solution was prepared in methanol (Panreac, Barcelona, Spain) at a concentration of 1 g/L and stored at 4 ºC. Working solutions were prepared by a rigorous dilution of the stock in Milli-Q ultrapure water (Millipore Corp., Madrid, Spain) or saliva blank samples, as required. Acetonitrile, ortophosphoric acid (both from Panreac) and Milli-Q ultrapure water were employed as components of the chromatographic mobile phase.

Sodium hydroxide solutions were used as acceptor phase while hydrochloric acid solution was employed for pH adjustment of the donor solution. 1-Octanol was used as supported liquid membrane (SLM) solvent. These reagents were purchased from Panreac. Sodium chloride from Sigma–Aldrich was used to adjust the ionic strength of the sample.

Polytetrafluoroethylene (PTFE) tape (100 μm in thickness, 0.5 μm of pore size) from Miarco (Valencia, Spain), polypropylene solid phase extraction cartridges (3 mL), pipette tips and PTFE top-caps, purchased from Analisis Vinicos (Tomelloso, Spain) were employed for the construction of the extraction units.

### 2.2. Samples collection and storage

Saliva samples were obtained from healthy volunteers who sometimes take paracetamol in order to avoid moderate pain and headache. It is important to point out that none of them were self-medicated and the dosages do not exceed the medical recommended values. In fact, volunteers just kindly provided the saliva samples following a specific protocol. Paracetamol was taken orally with 100 - 120 mL of water and the mouth was then rinsed with other 100 mL of water which was discarded in order to eliminate potential remains of paracetamol in the mouth. Saliva samples were collected using a citric acid solution as

stimulus. Food and liquids (apart from water) ingestion, chewing gum and tooth brushing were avoided 30 min before sampling [26]. The whole saliva (2.5-3 mL approximately) was spitting into a polypropylene commercial sterile pot (Deltalab, Barcelona, Spain) and immediately storage at -18 ºC. Before its extraction, the sample was thawed at room temperature and the saliva was centrifuged (J.P. Selecta, Barcelona, Spain) at 3800 rpm for 8 min [23]. No saliva dilution was required for sample extraction.

### 2.3. Apparatus

Chromatographic analyses were carried out using a HP1100 liquid chromatograph (Agilent, Palo Alto, CA, USA) equipped with a single wavelength photometer and a binary high-pressure pump for mobile-phase delivery. Data analysis was performed using HP ChemStation software. Chromatographic separation was achieved on a LiChrosorb $C_{18}$ (4.6 x 150 mm) column (Agilent, Palo Alto, CA, USA) under isocratic conditions. The mobile phase consisted of 80% of Milli Q water adjusted at pH 2.5 with ortophosphoric acid and 20% of acetonitrile. This chromatographic method was adapted from an early study [27]. Mobile phase was degassed in an ultrasonic bath model 3510 from Branson (Connecticut, USA) for 15 min (50 W, 60 Hz). The volume injected into the chromatograph for analysis was 10 µL. Separation was performed at room temperature. The flow rate was maintained at 1 mL/min and the analyte was monitored at 254 nm. Chromatographic peak area was chosen as analytical signal.

### 2.4. Extraction unit

A modification of the stir membrane liquid phase microextraction unit is carried out in order to make this device suitable for the analysis of volume-limited biological samples. The unit, as it is described elsewhere [20], is constructed using four commercial elements: (a) a PTFE top cap for SPE cartridges, (b) a section of a 3 mL-polypropylene SPE cartridge, (c) PTFE tape (100 µm in thickness, 0.5 µm of pore size) and d) an external element which is

cut from a pipette tip (1.2 internal diameter and 4 cm in height) where the small amount of biological sample is located.

The different building elements as well as their sizes and assembly process are depicted in **Figure 1**. The assembly process generates two different chambers (lower and upper) separated by a polymeric membrane which avoids their direct contact and mixing. The polymeric membrane, which was sealed by displacing the external element through the SPE cartridge section, is impregnated with 1-octanol to form a SLM which results essential in the liquid-liquid-liquid microextraction procedure. The lower chamber (ca. 50 µL) is filled with the acceptor alkaline phase while the upper one is filled with 2 mL of sample.

In this new proposal the height of the external element is increased, compared to the classic extraction unit, up to 4 cm generating an upper chamber where the sample can be located. The new proposal integrates the sample in the extraction device allowing to process lower sample volumes which was unfeasible with the older device. Moreover, this extraction unit can be simply agitated in a vortex. Various extraction units can be built in a reproducible way, thus permitting the simultaneous extraction of various samples. Moreover, each unit can be re-used several times just replacing the membrane and cleaning the elements with water.

### 2.5. Analytical procedure

First of all, the extraction unit is filled with 50 µL of a $10^{-2}$ M sodium hydroxide solution, which acted as acceptor phase. After that, the polymeric membrane is conveniently placed and the pores of the membrane are impregnated with 1-octanol (forming the SLM). Later on, the external element is placed and 2 mL of the aqueous standard or sample with the pH (2.5) and the ionic strength (300 g/L of sodium chloride) adjusted, are added into the upper chamber of the unit. Parafilm (Albus, Córdoba, Spain) is employed to cover the upper part of the extraction unit to avoid sample losses. Once ready, the extraction unit is placed on the vortex (Reax top, Heidolph) and stirred for 30 min. After this time, the sample is

withdrawn from the unit and the acceptor phase is collected using a 100 µL microsyringe and transferred to a HPLC vial. 10 µL of the extract are finally injected into the chromatograph for analysis. As different samples can be simultaneously extracted, the chromatographic run time marks the sample throughput which is of 6 samples per hour.

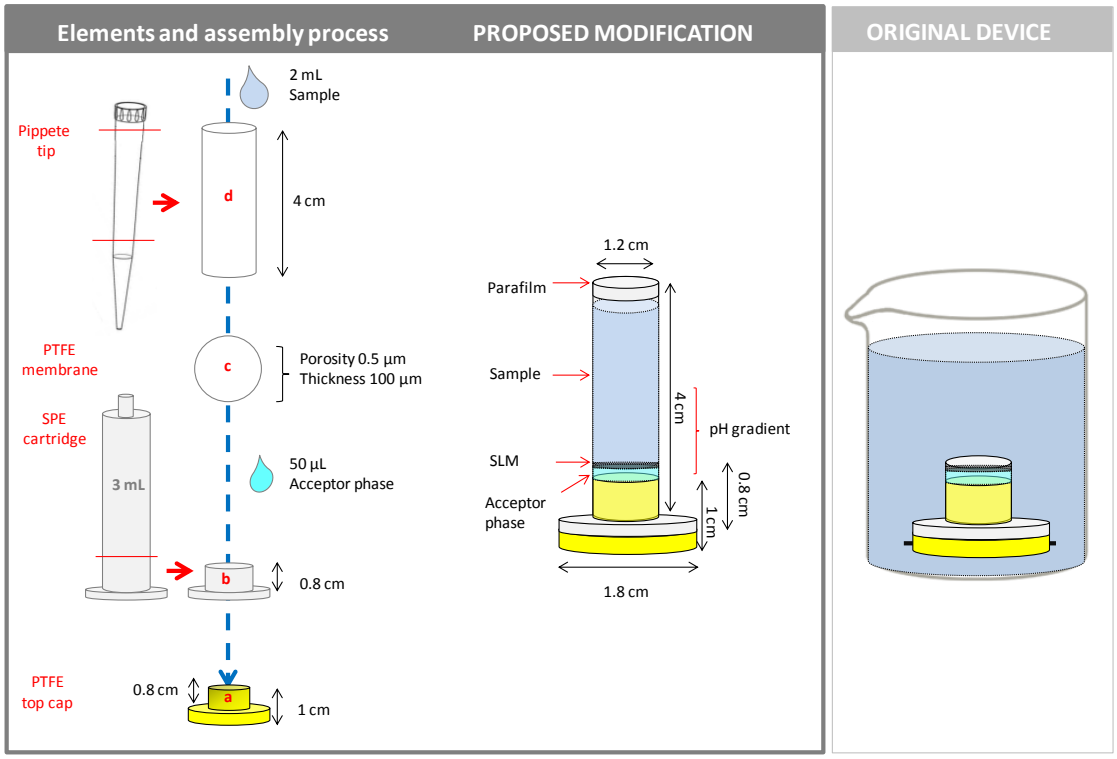


**Figure 1**. Description of the extraction device, the main elements employed in its construction and their assembly process. In addition the new proposal is compared to the conventional one.

## 3. RESULTS AND DISCUSSION

Three-phase LPME mode is especially appropriate for the extraction of paracetamol due to its chemical nature which presents a pH-dependent electrostatic charge. Paracetamol presents a net negative charge at alkaline pH while it is neutral in acid medium. Therefore, the pH gradient established at both sides of the SLM acts as the driving force of its transference from the acid samples to the alkaline acceptor phase. 1-octanol and dihexylether have been extensively used as SLM solvents in this type of applications. However, 1-octanol was finally selected as the optimum solvents since it provides higher extraction recoveries than dihexylether with hydrophilic drugs [28].

The rest of variables involved in the extraction process were optimized following a one variable at time approach and using the area of the chromatographic peak as analytical signal. **Table 1** resumes the variables considered, presenting also their initial values, the interval studied and the optimum values. The optimization process was performed by using standard solutions containing paracetamol at a concentration of 500 µg/L.

**Table 1**. List of the variables involved in the extraction process.

| Variable | Initial value | Interval studied | Optimum value |
|---|---|---|---|
| Sample pH | | 1-4 | 1-4 |
| Acceptor phase pH | 11 | 7 – 13 | $10^{-2}$ M NaOH |
| Ionic strength (% NaCl) | 0 | 0 – 30 | 30 |
| Sample volume (mL) | 0.5 | 0.5 – 2 | 2 |
| Extraction time (min) | 5 | 2 – 180 | 30 |

### 3.1. Sample and acceptor phase pH

Considering the acid-base properties of paracetamol (pKa = 9.5), the control of the pH in the acceptor and donor phases is a key parameter. The target compound has to be in its molecular form in the donor medium to be transferred to the SLM while the acceptor phase

should induce its ionization. Thus, the donor sample should be acid and the acceptor phase should be alkaline to establish an appropriate pH gradient at both sides of the SLM.

In this sense taking into account the reported chemical data obtained from SPARC on-line calculator [29], which is an on-line program that estimates several physico-chemical properties of organic compounds on the basis of their molecular structure, different donor phase´s pH (from 1 to 4) were evaluated. As negligible differences were observed in this interval, the donor phase (aqueous standard or sample) was adjusted with 10 µL of 1M hydrochloric acid solution to ensure an acid pH.

The pH of the acceptor phase was evaluated in the range of 7-13 by using different sodium hydroxide concentrations (**Figure 2**). The results showed how the analytical signal increases with the pH. According to the results, a $10^{-2}$ M sodium hydroxide concentration was selected as the optimum value since a reduction on the analytical signal is observed for higher concentrations.

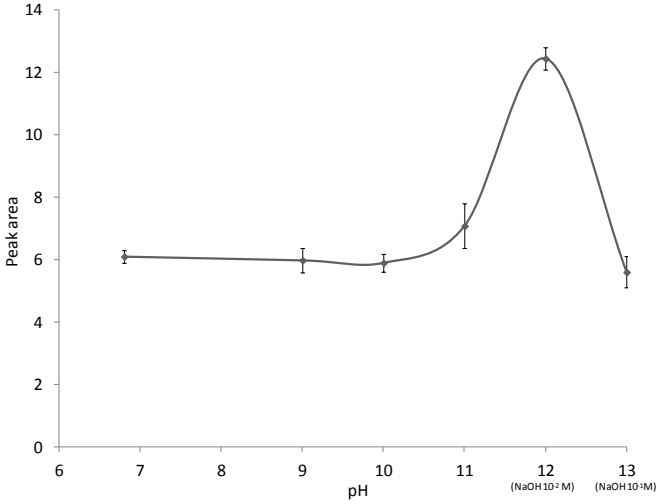


**Figure 2**. Effect of the acceptor phase pH on the extraction of paracetamol.

### 3.2. Effect of the ionic strength on the extraction

Ionic strength may play a key role in microextraction techniques. In fact, it may present two contradictory effects. On the one hand it can decrease the solubility of the target analyte in water, by the so-called salting-out effect, favoring its transference to the SLM. On the other hand, ionic strength may increase the viscosity of the sample, negatively affecting to the extraction kinetics. The influence of the ionic strength was evaluated in the range from 0 to 300 g/L using sodium chloride as model electrolyte. The peak area of the paracetamol increased with the electrolyte concentration (ca. 3 times, data not shown), showing a prevalence of the salting-out effect. Therefore, 300 g/L of sodium chloride was selected for subsequent experiments.

### 3.3. Effect of the sample volume on the extraction

The effect of the sample volume on the analyte extraction was evaluated in the range from 0.5 to 2 mL. No higher sample volumes were considered taking into account the volume-limitation of the samples. As it can be seen in **Figure 3**, the analytical signal increases with the sample volume. According to the obtained results, a sample volume of 2 mL was chosen as the optimal value to carry out the extraction procedure.

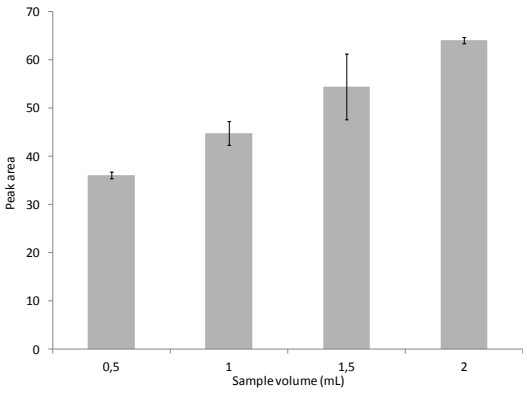


**Figure 3**. Effect of the sample volume on the extraction of paracetamol.

### 3.4. Effect of the extraction time on the extraction

The mass transfer of an analyte across the SLM in a three-phase system is based on passive diffusion. In this type of processes, the stirring of the sample may enhance the extraction rate as the transference of the analyte from the bulk sample to the extraction interface is promoted. This aspect was experimentally confirmed since the analytical signals were 4-5 times higher when vortex stirring was used compared with the static mode.

On the other hand, the extraction recovery depends on the extraction time and therefore this variable was evaluated in the range from 2 to 180 min. The results, which are depicted in **Figure 4**, showed an increase of the analytical signal with the time in the range from 0 to 60 min. The signal decreases with higher extraction times which can be ascribed to the opening of the unit when longer times are employed. In fact, the acceptor phase becomes slightly acid for extraction times longer than 60 min. Taking into account that the chromatographic lasts 10 min and the possible simultaneous extraction of three samples, 30 min was selected as the optimum value as a compromise between the sample throughput and sensitivity.

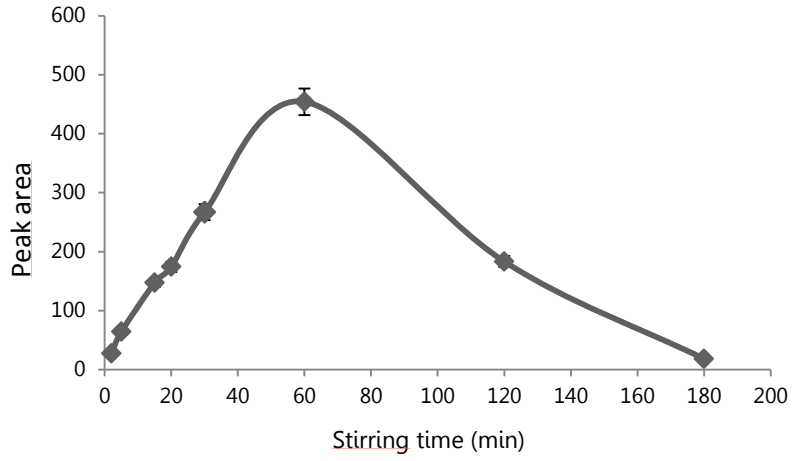


**Figure 4**. Effect of the stirring time on the extraction of paracetamol.

### 3.5. Analytical figures of merit

The analytical figures of merit of the proposed method are summarized in **Table 2**. Two calibration graphs (ex-matrix and in-matrix) for paracetamol were constructed in order to study the matrix effect. Ex-matrix calibration graph was constructed by using aqueous standards prepared at different concentrations while in-matrix calibration graph was constructed by extracting eight blank saliva samples spiked with the analyte in the same concentration range. By comparing the slopes of both calibration graphs a slightly decrease in the analytical signal was observed as a result of the matrix effect. Relative recoveries, which results to be 93±3, show a slight effect of the matrix on the extraction procedure.

The method was characterized on the basis of its linearity, sensitivity, precision and recovery using the in-matrix standard. Good linearity was obtained (R> 0.999). The limit of detection, which was calculated using a signal to noise ratio of 3, was 0.5 µg/L. The repeatability of the method, expressed as relative standard deviation (RSD) was found to be 14.2% for the septuplicate (n=7) analysis of a blank saliva sample spiked at 10 µg/L concentration level. These data are in agreement with the US FDA/Bioanalytical Method Validation Guidance which requires a precision lower than 15% and a minimum of five determinations [30].

A 10-fold preconcentration factor was obtained which involves an absolute recovery value of 25%. This absolute recovery is relatively high for a microextraction technique, since this type of procedures are non-exhaustive extraction methods, as only a fraction of the initial analyte is likely to be isolated [31].

The application of the proposed 3-phase stir membrane microextraction procedure to different human saliva samples was demonstrated by analyzing two different blank saliva samples spiked with paracetamol at two concentration levels (50 and 500 µg/L). A good accuracy was obtained since the absolute error of determination results to be 7%.

**Table 2**. Figures of merit of the proposed method for the determination of paracetamol.

| Analyte | *R* | LOD (µg/L) | LOQ (µg/L) | Linear range (µg/L) | Precision[a] (%) | Recovery[b] (%) | Accuracy[c] (%) |
|---|---|---|---|---|---|---|---|
| Paracetamol | 0.9999 | 0.52 | 1.7 | 1.7 - 10000 | 14.2 | 93±3 | 7 |

[a]Precision expressed as RSD; n=7.
[b]Average value obtained by comparison of recovery in real sample to standard aqueous samples.
[c]Average absolute error obtained for the analysis of different saliva samples spiked with the analytes at 50 and 500 µg/L.
*R*: Regression coefficient.

### 3.6. Paracetamol pharmacokinetics

Finally, the proposed method was applied to the determination of paracetamol in human saliva samples. The paracetamol pharmacokinetic study was carried out in three participants (2 males and 1 female). The obtained results are shown in **Table 3**, indicating some information about the dosage (amount), intake moment, and patient (sex, age, weight and height).

As it can be observed, the paracetamol concentration peak is observed at 30-120 min after its intake when it is taken after breakfast or lunch. However, the paracetamol peak is observed in the range from 10-30 min when it is taken on an empty stomach. Special attention should be given to patient II since he does not present zero level al initial time. This fact is due to the continuous treatment that he takes (380 mg of methocarbamol and 600 mg of paracetamol, every 8 hours) to solve a severe muscular and cervical problem. In spite of this combined treatment, no interferences were found in the chromatographic analysis of his saliva samples.

**Table 3**. Analysis of human saliva samples by the proposed method.

| | Patient data | | | | Intake timing | Dosage[a] | Concentration[b] Time[c] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sex | Age | Weight (kg) | Height (m) | | | Before | 10 | 30 | 60 | 120 | 240 |
| I | Male | 21 | 68 | 1.80 | After lunch | 650 | - | 0.25 | 2.42 | 1.79 | 1.36 | 0.82 |
| II | Male | 34 | 78 | 1.85 | After breakfast | 600 | 0.14 | 0.38 | 0.79 | 1.22 | 1.86 | 1.01 |
| III (i) | Female | 25 | 58 | 1.68 | Before breakfast | 500 | - | 6.37 | 6.73 | 3.66 | 2.50 | 1.54 |
| III (ii) | Female | 25 | 58 | 1.68 | After lunch | 650 | - | 0.15 | 0.20 | 2.82 | 4.33 | 2.88 |

[a]Amount of paracetamol taken via oral.
[b]Concentration of the drug in the sample.
[c]Sampling time after medication.

By way of an example, **Figure 5** shows the chromatograms obtained for the analysis of saliva samples from patient I at different sampling times after paracetamol intake.

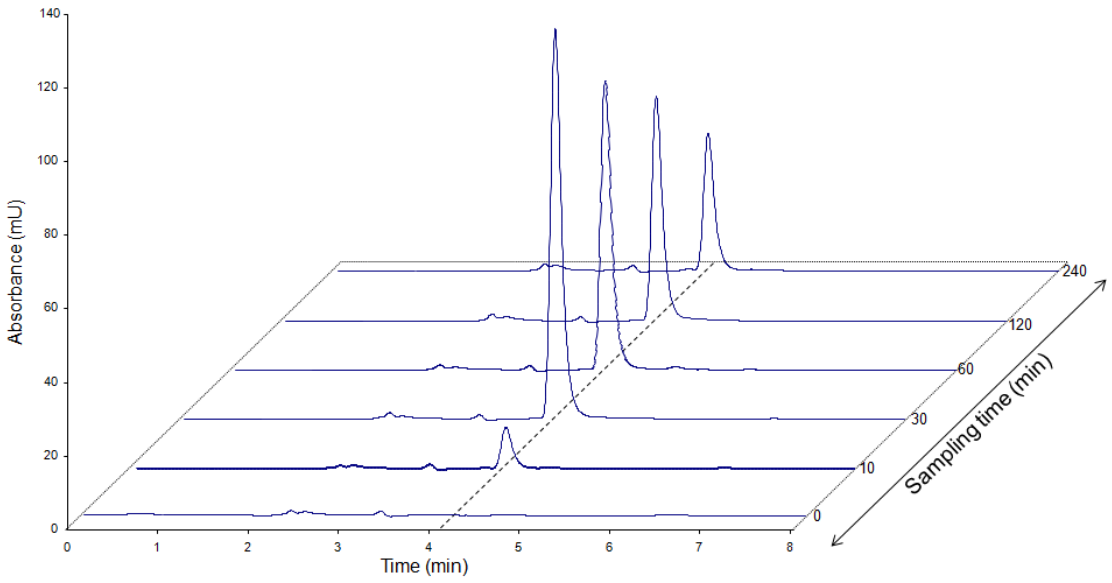


**Figure 5**. Chromatograms obtained for the analysis of saliva samples from patient I at different sampling times after paracetamol intake.

## 4. CONCLUSIONS

In the present article, an adaptation of the stir membrane liquid-liquid-liquid microextraction technique has been described. In this new proposal the height of the external element is increased, compared to the classic extraction unit, generating an upper chamber where the sample can be located. The new proposal integrates the sample in the extraction device allowing to process lower sample volumes which was unfeasible with the older device. Moreover, this extraction unit can be simply agitated in a vortex.. Furthermore, the unit continues being as cheap, simple and environmentally friendly as the conventional one. As a result, several units can be constructed being feasible the simultaneous extractions of several samples, what enhances the number of extraction that can be done.

The proposed extraction unit has been systematically optimized taking into consideration the main extraction variables and it has been evaluated using the determination of paracetamol in saliva as model analytical problem. The detection limit obtained in the low microgram per liter range allows the detection of paracetamol in a wide range of concentrations in human saliva samples.

## 5. FUTURE PERSPECTIVES

Extraction and stirring integrated techniques has been in a continuous evolution in the last years [32]. Among them, stir membrane both in the solid and liquid microextraction modes have found great application in environmental analysis. However, taking into consideration its inherent characteristics, stir membrane liquid phase microextraction be a promising tool for the isolation and preconcentration of drugs from biological samples including those which present a volume-limited availability. In this sense, further research should be performed in order to extend the applicability of this extraction device to others biological matrices such as plasma, blood, semen or sweat as well as to others ionizable analytes.

Nevertheless, automation is a weak point of the technique that limits its application in routine analysis. The potential automation of the technique, even its integration with analytical instruments, will be a future research line.

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CAPÍTULO 8

# Parallel artificial liquid membrane extraction of acidic drugs from human plasma

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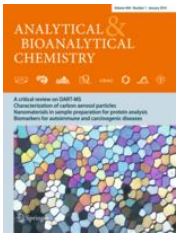


# Parallel artificial liquid membrane extraction of acidic drugs from human plasma

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The new sample preparation concept "Parallel artificial liquid membrane extraction (PALME)" was evaluated for extraction of the acidic drugs ketoprofen, fenoprofen, diclofenac, flurbiprofen, ibuprofen, and gemfibrozil from human plasma samples. Plasma samples (250 µL) were loaded into individual wells in a 96-well donor plate, and diluted with HCl to protonate the acidic drugs. The acidic drugs were extracted as protonated species from the individual plasma samples, through corresponding artificial liquid membranes each comprising 2 µL of dihexyl ether, and into corresponding acceptor solutions each comprising 50 µL of 25 mM ammonia solution (pH 10). The liquid membranes and the acceptor solutions were located in a 96-well filter plate, which was sandwiched with the 96-well donor plate during extraction. Parallel extraction of several samples was performed for 15 to 60 minutes, followed by high-performance liquid chromatography-ultraviolet detection of the individual acceptor solutions. Important PALME parameters including the chemical composition of the liquid membrane, extraction time, and sample pH were optimized, and the extraction performance was evaluated. Except for flurbiprofen, exhaustive extraction was accomplished from plasma. Linearity was obtained for all six drugs in the range 0.025-10 µg/mL, with $R^2$-values ranging between 0.998 and 1.000. Precision data were in the range 3-22% RSD, and accuracy data were within 72-130% with spiked plasma samples. Based on the current experiences, PALME showed substantial potential for future high-throughput bioanalysis of non-polar acidic drugs.

***Keywords***: *Parallel artificial liquid membrane extraction; Liquid-liquid extraction; 96-well; Supported liquid membrane; Human plasma; Acidic drugs.*

## 1. INTRODUCTION

The determination of drugs and drug metabolites in biological fluids is a challenge because biological fluids are complex matrices containing a large number of endogenous substances. Therefore, a sample preparation step is normally required prior to analysis of biological fluids by instrumental techniques like liquid chromatography (LC) or liquid chromatography-mass spectrometry (LC-MS). The sample preparation removes major matrix components, and improves the compatibility with the instrumental technique. This may also improve the specificity of the determination. In addition, the sample preparation can also improve the sensitivity of the determination due to pre-concentration.

Classic liquid-liquid extraction (LLE), solid phase extraction (SPE), and protein precipitation (PPT) have been widely used for sample preparation of biological samples [1]. In recent years, solid-phase microextraction (SPME) [2] and different liquid phase microextraction (LPME) formats [3-5] have attracted substantial attention, and in all formats the miniaturization of the sample preparation has dramatically reduced the consumption of hazardous organic solvent. Additionally, the different microextraction techniques have gained importance in bioanalysis as they can be applied to small volumes of biological samples.

Among the different liquid-phase microextraction formats explored, hollow fiber LPME (HF-LPME) has been especially popular for extraction from complex biological samples [4, 6-10]. In HF-LPME, target analytes are extracted from an aqueous sample, through a thin liquid membrane of an organic solvent immobilized in the pores in the wall of a porous hollow fiber, and into an acceptor solution located inside the lumen of the hollow fiber. While target analytes like drug substances easily transfer through the organic liquid membrane, most biological matrix components remain in the sample. Therefore, HF-LPME provides excellent clean-up from complex biological samples. In addition, because target analytes are extracted into a low µL volume of acceptor solution, HF-LPME can provide substantial enrichment. Finally, the consumption of organic solvent per sample is reduced to a few µL,

and consequently HF-LPME is an interesting green chemistry approach to sample preparation.

Although a large number of research papers have been published on HF-LPME, and the technique has been reviewed several times recently [6-10], the propagation of HF-LPME is still limited because no commercially available equipment is available and because HF-LPME is challenging to automate in a high-throughput configuration. To address this, the principles of HF-LPME were recently transferred and implemented into commercially available 96-well plates which were originally developed for filtration, and this new approach for liquid-phase microextraction was termed "parallel artificial liquid membrane extraction" (PALME) [11]. In PALME µL volumes of samples are loaded into individual wells in a 96-well donor plate, and target analytes are extracted from the individual samples, through corresponding liquid membranes each comprising a few µL of organic solvent, and into corresponding acceptor solutions each comprising a µL volume of aqueous solution.

The liquid membranes and the acceptor solutions are located in a 96-well filter plate with disk-type membranes, which is sandwiched with the 96-well donor plate and agitated during extraction. In a recent paper, PALME was successfully used for the extraction of selected basic drugs (pethidine, nortriptyline, methadone and haloperidol) from human plasma [11]. Extraction recoveries up to 74% and RSD-values below 12% were reported, and up to 96 samples were extracted simultaneously in 30 min. PALME provided excellent sample clean-up, required only 2 µL of organic solvent per sample (green chemistry), and is definitely amenable to future automation and high-throughput operation. Further development of PALME is expected in the near future, but for this to be successful, fundamental understanding and more experimental data are required.

The extraction principle of PALME is similar to HF-LPME, but volumes, phase ratios and device geometries are totally different. Therefore, more experimental data with PALME, and comparison with existing HF-LPME data, is required before HF-LPME experiences accumulated in the literature since 1999 can be translated to PALME. The current paper is

intended to give this type of experimental data, and is focused on the extraction of selected non-polar acidic drugs used as model analytes. Optimization of operational parameters for the acidic analytes, exhaustive extraction from plasma, and comparison with previous HF-LPME on the same acidic analytes are highlighted in this report.

## 2. EXPERIMENTAL SECTION

### 2.1. Chemicals

Ketoprofen, fenoprofen, diclofenac, flurbiprofen, ibuprofen, and gemfibrozil were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock standard solutions of each analyte were prepared in methanol (Sigma–Aldrich) at a concentration of 1 mg/mL. The stock solutions were protected from light and stored at +4°C. The stock solutions were used for spiking 10 mM HCl or drug-free human plasma and these were employed as sample solutions.

2-Hexyl-1-decanol, 1-nonanol, 1-octanol, 2,2-dimethyl-1-propyl benzene, and dihexyl ether were purchased from Sigma-Aldrich. Dodecyl acetate and 2-nitrophenyl octyl ether were from Fluka (Buchs, Switzerland). Isopentyl benzene was from Tokyo Chemical Industry (Tokyo, Japan). All these organic solvents were evaluated to form the liquid membrane.

Hydrochloric acid (36%) and ammonia solution (25%) were purchased from Merck (Darmstadt, Germany) and they were employed to adjust the pH in the sample and acceptor solutions, respectively. Acetonitrile and formic acid (Merck) were used as components of the mobile phases. Purified water was obtained from a Millipore Milli-Q water purification system (Millipore, MA, USA).

### 2.2. Biological matrices and sample preparation

Drug free human plasma was obtained from Oslo University Hospital (Oslo, Norway). The samples were stored at -32°C. 125 µL of plasma samples were spiked with the stock

standard solutions containing the target analytes and mixed with 125 µL solution of 250 mM HCl.

### 2.3. PALME set-up and analytical procedure

The PALME set-up was described recently [11] and comprised four commercial elements: (a) 96-well donor plate of polypropylene with 0.5 mL wells from Agilent (Santa Clara, CA, USA), (b) 0.2-mL thin-walled 8 tubes strips from Thermo Scientific (San Diego, CA, USA) for preparation of the 96-well filter plate, (c) flat porous polypropylene membrane with 100 µm thickness and pore size 0.1 µm (Accurel PP 1E R/P, Membrana, Wuppertal, Germany), and (d) a lid to avoid potential losses of the acceptor solution by evaporation. The flat porous polypropylene membrane was sealed to the open end of the thin-walled tubes using a Cotech soldering iron station (Clas Ohlson AB, Insjon, Sweden) at 185°C for 2 s. The closed end of the thin-walled tubes was cut off, thus creating a chamber in which the acceptor solution is located. A complete 96-well filter plate comprised 12 individual 8 tube strips.

The different building elements, as well as the assembly process, are depicted in **Figure 1**. First, samples of 250 µL were pipetted into the 96-well donor plate. The samples (250 µL) were either 10 mM HCl containing the target analytes or plasma samples diluted in 250 mM HCl (1:1, v/v). After that, 2 µL of organic solvent was pipetted into the porous polypropylene membrane to form the liquid membrane. The small volume of organic solvent rapidly permeated into the pores of the polypropylene membrane and was immobilized by capillary forces in less than 1 min. Subsequently, the acceptor wells with the SLM were located above the donor plate and 50 µL of 25 mM ammonia solution (pH 10) was pipetted into the acceptor wells, acting as the acceptor solution. The choice of the acceptor solution was based on compatibility with liquid chromatography. The whole assembly was agitated on a vibrating platform shaker (Vibramax 100, Heidolph Instruments, Schwabach, Germany) at 900 rpm for the predetermined time to perform the PALME process. After PALME, the acceptor solutions were collected using a micropipette and

transferred to HPLC vials. Twenty µL of the extract was finally analyzed by liquid chromatography.

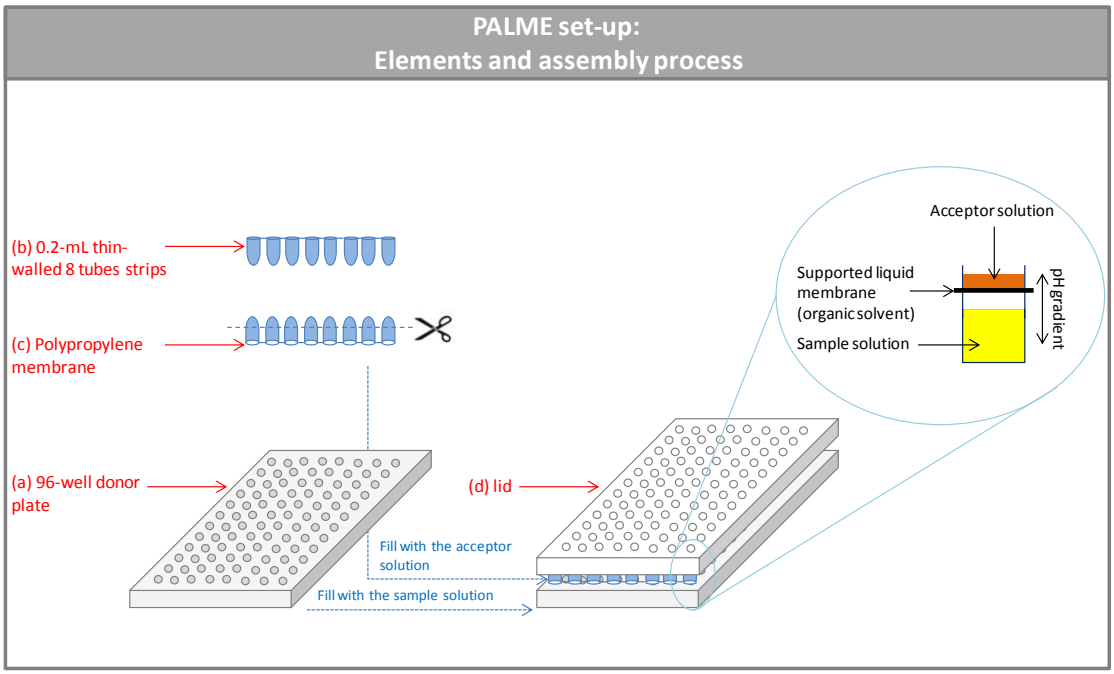


**Figure 1**. Parallel artificial liquid membrane extraction set-up.

## 2.4. Liquid chromatography

Liquid chromatography was carried out using an Agilent 1200 Series HPLC system with UV-detection from Agilent Technologies (Santa Clara, CA, USA) equipped with Micro Vacuum Degasser, Binary Pump SL, Autosampler, Column Compartment /Column Oven, and a Diode Array and Multiple Wavelength Detector SL operated at 220 and 254 nm, respectively. Data acquisition was performed using HP ChemStation software (Agilent Technologies).

Chromatographic separation was achieved on a Syncronis $C_{18}$ column (3 µm particle size; 3 mm x 100 mm) (Thermo Fisher Scientific, Waltham, MA, USA) maintained at 30°C.

The mobile phases consisted of (A) 0.1% (v/v) formic acid (pH 2.7) and (B) acetonitrile with 0.1 % (v/v) formic acid. The flow rate was set to 0.5 mL/min. The injection volume was 20 µL. The initial composition was fixed at 50% B, the percentage being increased to 75% in 5 min and kept constant for 3 min. Between each injection an equilibration time of 2 minutes was used.

## 3. RESULTS AND DISCUSSION

The acidic drug substances ketoprofen, fenoprofen, diclofenac, flurbiprofen, ibuprofen, and gemfibrozil were selected as model analytes. These drug substances were chosen because of their non-polar character (log P>3) and because they have been successfully extracted by HF-LPME in earlier work [12-17]. The concentration of each model analyte was 1 µg/mL, and this was equivalent to therapeutic levels typically found in biological samples [18].

In the first paper devoted to PALME [11], operational parameters such as sample volume, acceptor solution volume, and agitation rate were studied and optimized in depth. Based on this previous experience, the sample volume was fixed to 250 µL, the acceptor volume was fixed to 50 µL, and the agitation rate was fixed to 900 rpm in the current work. During the extraction process, the target analytes were extracted in their neutral state from the acidic sample solution (10 mM HCl or acidified plasma), through the liquid membrane (organic solvent), and into the alkaline acceptor solution ($NH_3$) where deprotonation took place. Following deprotonation, the model analytes were prevented from back-extraction into the liquid membrane. The acceptor solutions were finally analyzed by liquid chromatography with ultraviolet detection.

### 3.1. Selection of liquid membrane

Because PALME was performed for acidic drug substances for the first time, the nature of the liquid membrane was optimized. Nine different organic solvents were tested as liquid membrane candidates, namely 1-hexyl-1-decanol, 1-nonanol, 1-octanol, 1-decanol, dodecyl acetate, 2-nitrophenyl octyl ether, 2,2-dimethyl-1-propyl benzene, isopentyl benzene, and dihexyl ether. These solvents were selected based on related experience from HF-LPME of acidic drugs [19]. In all cases, 2 µL of the organic solvent was pipetted into the porous polypropylene membrane. This volume of solvent provided a spot of similar size as the diameter of the sample and acceptor wells (6 mm) and covered the entire cross-sectional area of the porous polypropylene membrane.

Extraction recoveries as well as the relative standard deviation (RSD) were evaluated from quadruplicate (n=4) experiments with each solvent after 30 min of PALME, and the results are shown in **Table 1**. The relatively long extraction time was chosen to make sure that extraction equilibrium was obtained. Water solubility (computer calculated values) is also listed for all the solvents. Low water solubility is mandatory to avoid leakage of the liquid membrane into the sample. As it can be seen from the data, the extraction performance and the RSD values were influenced by the type of the organic solvent.

Among the solvents tested in this work, the most successful were 2,2-dimethyl-1-propyl benzene, isopentyl benzene, and dihexyl ether, which provided exhaustive extraction from 10 mM HCl samples and which provided the lowest RSD values.

**Table 1**. Parallel artificial liquid membrane extraction recoveries with different organic solvents as artificial liquid membrane.

| Organic solvent | Water solubility[a] (µg/mL) | Absolute recovery (%)[b,c] (% RSD) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Ketoprofen | Fenoprofen | Diclofenac | Flurbiprofen | Ibuprofen | Gemfibrozil |
| 2-Hexyl-1-decanol | 0.039 | 34 (2) | 36 (10) | 32 (15) | 29 (16) | 35 (14) | 37 (12) |
| 1-Nonanol | 390 | 63 (2) | 51 (7) | 45 (6) | 49 (8) | 49 (6) | 45 (7) |
| 1-Octanol | 1200 | 78 (9) | 59 (12) | 51 (14) | 46 (14) | 54 (15) | 49 (17) |
| 1-Decanol | 120 | 79 (7) | 74 (7) | 74 (8) | 71 (8) | 79 (5) | 79 (7) |
| Dodecyl acetate | 20 | 99 (4) | 101 (5) | 88 (1) | 84 (3) | 97 (5) | 88 (6) |
| 2-Nitrophenyl octyl ether | 6 | 89 (11) | 98 (6) | 96 (8) | 93 (11) | 100 (6) | 102 (12) |
| 2,2-Dimethyl-1-propyl benzene | 1.9 | 88 (1) | 107 (1) | 102 (3) | 94 (4) | 103 (2) | 108 (3) |
| Isopenthyl benzene | 2.5 | 99 (4) | 103 (1) | 105 (5) | 104 (3) | 104 (1) | 112 (3) |
| Dihexyl ether | 110 | 105 (2) | 110 (3) | 105 (2) | 101 (2) | 106 (2) | 111 (3) |

[a]Data obtained from SciFinder, at 25°C and pH 10.
[b]n = 4.
[c]Drug concentration: 1 µg/mL

### 3.2. Selection of extraction time

In a subsequent set of experiments, the extraction time was studied in the range from 2 to 30 min with 2,2-dimethyl-1-propyl benzene, isopentyl benzene, and dihexyl ether as liquid membranes. PALME was performed from 10 mM HCl samples. The aim was to study the extraction kinetics of the target compound across different liquid membranes.

Extraction recoveries versus extraction time are depicted in **Figure 2**. With 2,2-dimethyl-1-propyl benzene and isopentyl benzene as liquid membrane, 30 min was required to extract ketoprofen exhaustively, whereas the other drug substances were extracted exhaustively

after 15 min. On the other hand, with dihexyl ether even ketoprofen was extracted exhaustively after 15 min. Due to this observation, dihexyl ether was selected as the optimal liquid membrane, and the extraction time was set to 15 min from 10 mM HCl samples.

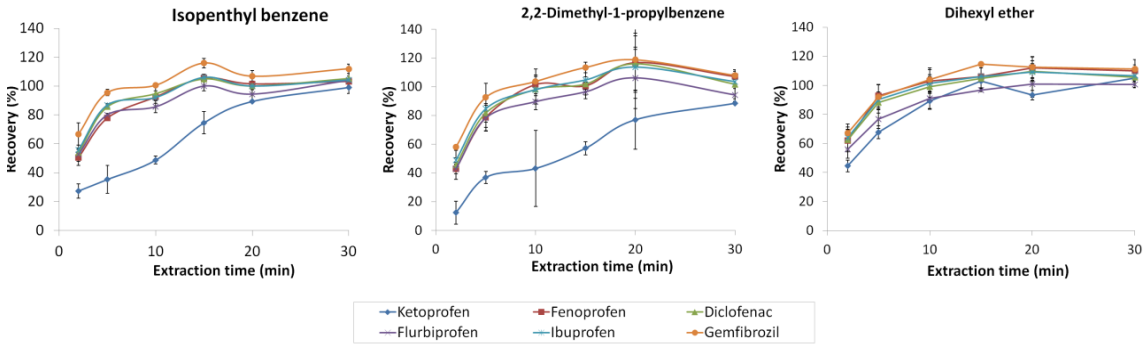


**Figure 2**. Parallel artificial liquid membrane extraction recovery versus extraction time from 10 mM HCl (sample volume 250 µL; acceptor volume 50 µL; n = 4. Three organic solvents were employed as liquid membrane (A) isopentyl benzene, (B) 2,2-dimethyl-1-propylbenzene, and (C) dihexyl ether.

### 3.3. Selection of sample pH

For efficient PALME of the acidic drugs, the sample should be acidic and the acceptor solution should be alkaline to establish an appropriate pH gradient across the liquid membrane. The effect of sample pH in PALME was initially tested from water samples mixed with different buffers in the pH range 2.0 to 7.0 (data not shown). Negligible differences were observed in the pH range from 2.0 to 4.0, and the extraction recoveries were between 80 and 100%. However, the extraction efficiency decreased markedly with increasing pH in the range from 4.0 to 7.0. These results are in line with common LLE theory, where partly ionized analytes are extracted to a less degree compared to non-ionized analytes. When pH in the sample reached 4.0, it was close to the pKa of the acidic model analytes and hence a partly ionization occurred. The extraction efficiency therefore decreased with increasing pH.

### 3.4. Extraction from plasma

The effect of sample pH was also studied with spiked plasma samples. Thus, spiked plasma was diluted 1:1 (v/v) with HCl in the concentration range from 10 mM to 1 M to test acidification. The results (data not shown) were similar to the results obtained from pure water samples. Mixing plasma with 10 mM HCl, pH was still above 4.5 due to the buffer capacity of plasma, and extractions were inefficient. However, by increasing the HCl concentration, pH in the plasma samples decreased, and at pH 4.0 or below, recoveries stabilized in the range 45-75%. Clearly, the recoveries were lower from plasma than pure water samples and this was attributed to the protein binding of the drugs.

Based on this experience, plasma samples were diluted 1:1 with 250 mM HCl to a final HCl concentration of 125 mM prior to PALME. In order to improve extraction recoveries from plasma, the extraction time was re-investigated using spiked plasma samples. The influence of the extraction time was now evaluated in the range from 15 to 120 min as illustrated in **Figure 3**. Clearly, extraction recoveries increased up to 60 min, and except for flurbiprofen, exhaustive extraction was obtained from plasma after 60 min.

A comparison of **Figure 2** and **Figure 3** demonstrated that the extraction kinetics was significantly influenced by the biological matrix, and therefore 60 min was selected as optimal extraction time from plasma.

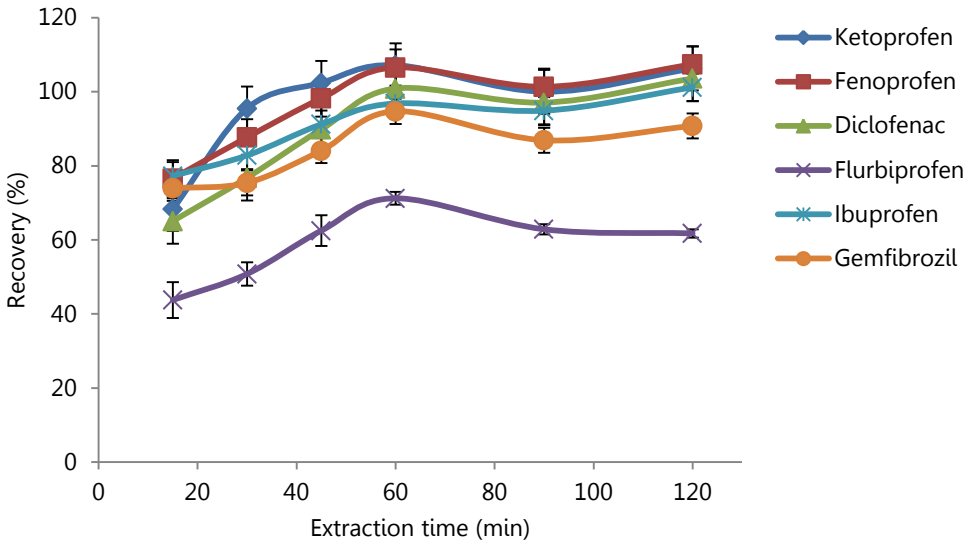


**Figure 3.** Parallel artificial liquid membrane extraction recovery versus extraction time from plasma (sample volume 250 µL; acceptor volume 50 µL; n = 4). Sample dilution 1:1, plasma: 250 mM HCl, v/v. Dihexyl ether was employed as liquid membrane.

### 3.5. Experiences with a polypropylene membrane and a polyvinylidene fluoride membrane

In the first PALME paper reported recently [11], the initial experiments were carried out with a polyvinylidene fluoride (PVDF) membrane as the porous solid support for the liquid membrane. However, nonspecific binding of the alkaline drug substances to the PVDF membrane was observed, as reflected by non-linearity in the evaluation data. Therefore, the PVDF membrane was replaced with a porous polypropylene (PP) membrane [11], exactly the same membrane as used above for PALME of acidic drugs.

In a next series of experiments, both PP and PVDF membranes were tested as porous support for PALME of acidic drugs. First, the linearity was checked in the range of 0.025 to 10 µg/mL from diluted plasma samples (1:1, v/v, with 250 mM HCl) spiked with the acidic

analytes. Each level of concentration was evaluated in quadruplicate. With the PP membrane, excellent linearity was obtained and the precision, expressed as relative standard deviation (RSD), was better than 8%. Thus, the PP membrane showed no tendency for nonspecific binding of the model analytes. With the PVDF membrane, the RSD values increased up to 40% and deviation from linearity was clearly observed. Obviously, the PVDF membrane also suffered from non-specific binding of the acidic analytes. The experiments with the PVDF membrane were repeated after washing the membrane with ethanol and acetone, but still non-specific binding was observed. Consequently, the PVDF membrane was found to be inappropriate also for the non-polar acidic drugs, and all remaining experiments in this report were conducted with the porous PP membrane.

### 3.6. Evaluation

The calibration curves for the target analytes were constructed by using nine diluted plasma samples (1:1, v/v, with 250 mM HCl) where each of the samples were spiked with the six target analytes at controlled concentration levels. The plasma samples were subjected to the optimized PALME procedure combined with liquid chromatography ultraviolet detection in quadruplicate. Typical chromatograms are illustrated in **Figure 4**. The upper chromatogram illustrates PALME followed by liquid chromatography with ultraviolet detection at 220 nm from a blank human plasma sample. The lower chromatogram illustrates a similar analysis of a human plasma sample spiked with 500 ng/mL of each of the acidic drugs.

The analytical figures of merit of the proposed method are summarized in **Table 2.** The method was characterized on the basis of its linearity, absolute recovery, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy. As seen from the data, linearity was obtained with $R^2$ values between 0.997 and 1.000 in the respective concentration ranges. The LODs, calculated using a signal-to-noise ratio (S/N) of 3, were found to be in the range from 6.4 to 22.9 ng/mL. The LOQs (S/N ratio of 10) were in the range from 22.1 to 75.0 ng/mL. All the LOQs were well below the lowest concentrations recommended for

therapy, and were therefore considered as fully acceptable. These results are also summarized in **Table 2**. **Figure 5** shows the chromatogram obtained after PALME from plasma spiked with the concentration close to the LOQs (50 ng/mL).

**Table 2**. Analytical figures of merit.

| Drug substance | pKa[a] | Log P[a] | Therapeutic range (µg/mL)[b] | Linear range[c] (µg/mL) | $R^2$ | LOD[f] (ng/mL) | LOQ[g] (ng/mL) |
|---|---|---|---|---|---|---|---|
| Ketoprofen | 3.88 | 3.61 | 1-6 | 0.050-10 | 0.998[c] | 10.0 | 33.0 |
| Fenoprofen | 3.96 | 3.65 | 30-60 | 0.025-10 | 0.998[d] | 6.4 | 21.1 |
| Diclofenac | 4.00 | 4.26 | 0.5-3 | 0.050-10 | 1.000[c] | 11.2 | 37.0 |
| Flurbiprofen | 4.42 | 3.94 | 5-15 | 0.050-10 | 0.999[c] | 12.9 | 42.5 |
| Ibuprofen | 4.85 | 3.84 | 15-30 | 0.050-10 | 0.999[c] | 10.1 | 33.3 |
| Gemfibrozil | 4.42 | 4.39 | 25 | 0.100-10 | 0.997[e] | 22.9 | 75 |

[a]Collected from www.chemicalize.org
[b]Collected from [18].
[c]Eight concentration levels.
[d]Nine concentration levels.
[e]Seven concentration levels.
[f]*LOD*, limit of detection, calculated based on a signal-to-noise ratio of 3.
[g]*LOQ*, limit of quantification, calculated based on a signal-to-noise ratio of 10.

Next, the precision of the method was tested and the results are listed in **Table 3**. The precision, expressed as relative standard deviation (RSD), was evaluated under intra-day and inter-day conditions using human plasma samples spiked at three different concentrations: 50, 100, and 500 ng/mL. Intra-day precision (% RSD, n=6) at 50, 100, and 500 ng/mL ranged from 3-15%, 4-10%, and 3-9% RSD, respectively. Inter-day precision (% RSD) was evaluated on 3 consecutive days (each day a new sample was spiked, extracted and analyzed). Inter-day precision was better than 15% in all cases, except for flurbiprofen when tested at 500 ng/mL. Thus, all the data, except for flurbiprofen at 500 ng/mL, were within the acceptance criteria for bioanalytical method validation, which should not deviate

more than 15% (and not more than 20% at the LOQ) [20]. At this initial stage, where PALME was performed with home-built acceptor well plates, the evaluation data were considered as acceptable.

Finally, the accuracy of the method was evaluated using human plasma samples. First, six blank plasma samples were analyzed in order to verify the absence of the target analytes. Then the blank plasma samples were spiked to 50, 100, and 500 ng/mL, respectively, and were analyzed by the proposed method. The corresponding accuracy data are reported in **Table 3**, and were in the range of 79 to 130%.

The absolute recoveries were calculated at the low, middle and high concentration levels, and the results are given in **Table 3**. For most of the analytes, exhaustive extraction was achieved, and the absolute recoveries were found to be independent of the drug concentration.

**Table 3**. Accuracy and precision.

| Drug substance | Spiked (ng $mL^{-1}$) | Absolute recovery (%) | Intra-day | | Inter-day | |
|---|---|---|---|---|---|---|
| | | | RE (%) | RSD (%) | RE (%) | RSD (%) |
| Ketoprofen | 50 | 91 | 87 | 5 | 93 | 14 |
| | 100 | 93 | 109 | 4 | 107 | 14 |
| | 500 | 81 | 93 | 3 | 102 | 8 |
| Fenoprofen | 50 | 99 | 105 | 3 | 95 | 12 |
| | 100 | 107 | 115 | 4 | 104 | 9 |
| | 500 | 95 | 89 | 5 | 92 | 3 |
| Diclofenac | 50 | 83 | 79 | 6 | 78 | 7 |
| | 100 | 97 | 103 | 9 | 95 | 14 |
| | 500 | 90 | 84 | 8 | 89 | 5 |
| Flurbiprofen | 50 | 59 | 96 | 11 | 100 | 10 |
| | 100 | 63 | 112 | 7 | 116 | 4 |
| | 500 | 58 | 84 | 9 | 103 | 22 |
| Ibuprofen | 50 | 95 | 130 | 6 | 124 | 7 |
| | 100 | 105 | 126 | 6 | 115 | 9 |
| | 500 | 84 | 86 | 7 | 90 | 3 |
| Gemfibrozil | 50 | 108 | 108 | 15 | 91 | 13 |
| | 100 | 89 | 79 | 10 | 120 | 4 |
| | 500 | 92 | 72 | 8 | 83 | 8 |

Intra-day: mean of 6 determinations in the same day.
Inter-day: mean of 3 different days.
RE (Relative error) = (nominal – found)/nominal *100.
RSD (Relative standard deviation) = (standard deviation /mean)*100.

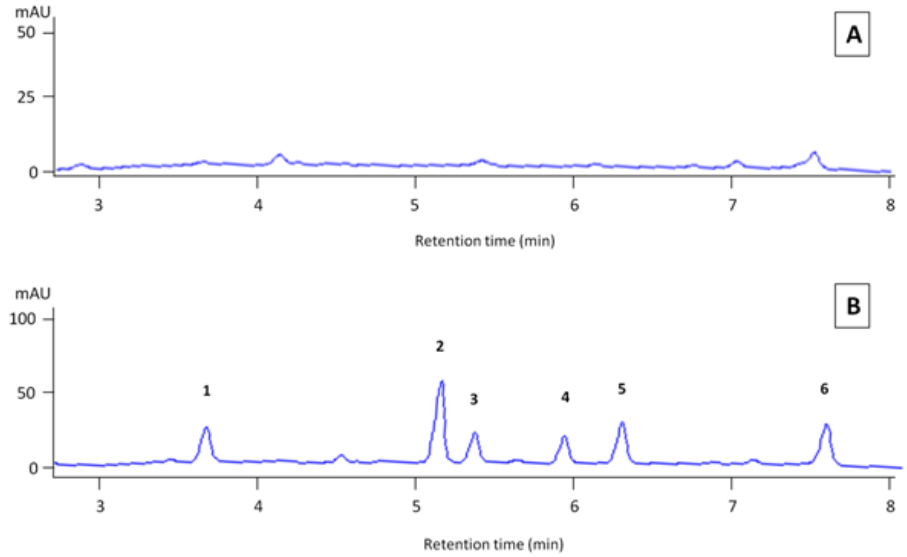


**Figure 4.** Liquid chromatography with ultraviolet detection (220 nm) after parallel artificial liquid membrane extraction from A) blank human plasma and B) human plasma spiked with 500 ng/mL. (1) Ketoprofen, (2) fenoprofen, (3) diclofenac, (4) flurbiprofen, (5) ibuprofen and (6) gemfibrozil.

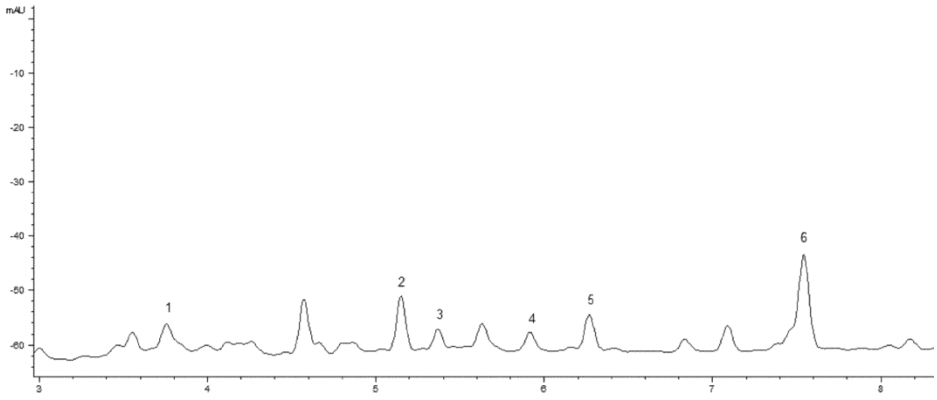


**Figure 5.** Chromatogram obtained from parallel artificial liquid membrane extraction from human plasma spiked to a concentration of 50 ng/mL. (1) Ketoprofen, (2) fenoprofen, (3) diclofenac, (4) flurbiprofen, (5) ibuprofen and (6) gemfibrozil.

### 3.7. Comparison with experiences from HF-LPME

In several papers, the NSAIDs ibuprofen, ketoprofen, naproxen, and diclofenac [12-17] have been extracted by HF-LPME. Although the reported set-up for HF-LPME differed significantly from the current PALME set-up in terms of volumes, phase ratios, and geometries, the experimental conditions reported with HF-LPME are very close to the optimal PALME conditions in this report. This indicates that experimental conditions from HF-LPME can be transferred directly to PALME without major modifications. Dihexyl ether has been used as the liquid membrane in HF-LPME [12-17], and this liquid was superior also for PALME based on the experiences reported above. Dihexyl ether is characterized by low polarity-polarizability, moderate hydrogen-bond basicity, and zero hydrogen-bond acidity [21]. However, the current work has revealed that also two different aromatic hydrocarbons with moderate polarity-polarizability, low hydrogen-bond basicity, and zero hydrogen-bond acidity were efficient for extraction of the selected non-polar acidic drugs. The latter type of solvents has not been used for HF-LPME of NSAIDs. The pH conditions used in the PALME was very similar to those used in HF-LPME, with pH 2-4 in the sample, and with strongly alkaline conditions in the acceptor solution using either NaOH or alkaline buffers.

Most HF-LPME papers related to NSAIDs have been focused on extraction from large sample volumes in the range 50 to 100 mL [13-17]. Therefore, comparison of current performance data from PALME with HF-LPME is difficult. However, in one HF-LPME paper, ibuprofen, naproxen, and ketoprofen were extracted from 2.5 mL water and urine [12], and these data can to some extent be used for comparison. In the HF-LPME report, the equilibrium extraction time form pure water was 30-45 minutes, while only 15 minutes was required in the current work. The HF-LPME equilibrium recovery for ibuprofen was comparable with data reported in the present study, whereas recoveries for naproxen and ketoprofen were slightly lower in HF-LPME than in PALME.

## 4. CONCLUSIONS

In the present work, selected non-polar acidic drugs were extracted from human plasma by parallel artificial liquid membrane extraction (PALME). Several samples were extracted in parallel, using a combination of a 96-well donor plate and a 96-well filter plate sandwiched together. The acidic drugs were extracted as protonated species from the individual plasma samples, through corresponding artificial liquid membranes each comprising 2 µL organic solvent, and into corresponding acceptor solutions (ammonia). Although the acidic drugs were highly bound to proteins in plasma, the drugs (except for flurbiprofen) were extracted exhaustively from plasma during 60 minutes of PALME. Evaluation experiments supported that PALME provided linear, repeatable, and accurate analytical data from plasma samples. The optimal experimental conditions for PALME were very similar to those reported in the literature for the same acidic drugs by hollow fiber liquid-phase microextraction (HF-LPME). This indicates that important HF-LPME experiences accumulated in the literature since 1999 can be transferred directly to PALME in the future for optimal performance.

The main advantages of PALME, as compared to HF-LPME, include the possibility for high-throughput operation, the use of commercially available equipment, and the great potential for automation. On the other hand, sample volumes are limited in PALME, and therefore the technique is mostly suited for bioanalysis and other application areas where sample volumes are limited (< 1 mL).

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BLOQUE V

# RESULTADOS Y DISCUSIÓN

En este apartado de la Memoria de Tesis Doctoral se resumen los resultados más relevantes obtenidos a lo largo del desarrollo experimental de la misma. El objetivo es ofrecer un enfoque integral de las diferentes herramientas analíticas desarrolladas.

La investigación que constituye la parte principal de la Tesis está enmarcada dentro del tratamiento de muestra y tiene como denominador común la integración de la agitación en la extracción para mejorar la cinética de los procesos.

Esta sección se ha dividido en dos bloques en función de las configuraciones empleadas:

- **Unidades de extracción/agitación basadas en discos.** En este apartado se abordarán las metodologías propuestas que emplean discos como material extractante o bien como elemento de soporte para llevar a cabo sobre ellos procesos de síntesis.
- **Unidades de extracción/agitación basadas en el empleo de membranas.** En este apartado se describirán los diseños propuestos que hacen uso de membranas, bien actuando como medio extractante o como separadoras de fases.

Con el fin de facilitar la lectura y reflexión de lo que se va a exponer, estos dos grandes apartados se dividirán a su vez en apartados más específicos. Finalmente, en un tercer bloque se comparan todas las metodologías propuestas.

**Generalidades de las técnicas de microextracción**

La determinación de compuestos a baja concentración implica su aislamiento de la muestra y preconcentración antes de su análisis. Tradicionalmente, la separación y preconcentración se ha logrado mediante el empleo de técnicas tales como la extracción líquido-líquido (*liquid-liquid extraction*, LLE) y la extracción en fase sólida (*solid phase extraction*, SPE). Generalmente, estos métodos consumen grandes cantidades de muestra y disolventes, y requieren de mucho tiempo y dedicación por parte del analista.

La evolución hacia técnicas de extracción miniaturizadas en las que se reduzca el consumo de disolventes orgánicos ha dado lugar a una nueva aproximación en la preparación de muestras dentro del contexto de la Química Analítica, con una tendencia clara hacia la automatización, miniaturización y simplificación. Sin duda, la etapa de pretratamiento es la que más esfuerzos investigadores demanda a la hora de llevar a cabo una reducción de dimensiones en el tratamiento de las muestras. La miniaturización de las operaciones previas es de especial relevancia cuando se trabaja con muestras que son valiosas, o de las que sólo se dispone de cantidades pequeñas. Además, esta miniaturización reduce también el coste del análisis por el menor consumo de reactivos y disolventes y la reducción del volumen de residuos que deben ser gestionados.

No obstante, para poder trabajar a esa escala reducida, resulta imprescindible disponer de materiales (sólidos sorbentes y líquidos extractantes) cuya capacidad de extracción supere en eficiencia a los materiales convencionales, ya que de otro modo resulta inviable obtener factores de enriquecimiento elevados utilizando cantidades de sólidos o líquidos en el entorno de los micro/nano gramos o micro/nano litros.

Los dos factores asociados a las técnicas de microextracción que tienen un papel fundamental son:

- La *termodinámica* de la extracción, que determina la cantidad máxima de analito que puede extraerse por el sorbente bajo unas determinadas condiciones (depende por tanto de la capacidad sorbente del material extractante).

- La *cinética* de la extracción, que determina la velocidad a la que se extraen los analitos y el tiempo necesario hasta que se alcance el equilibrio. Ya que la mayoría de las técnicas de extracción tienen lugar bajo condiciones de difusión controlada, la agitación favorece la difusión de los analitos desde el seno de la muestra hasta la fase extractante. Además, mediante la agitación se reduce el espesor de la capa de Nernst que es clave en el control cinético del proceso. Esta agitación puede

llevarse a cabo mediante un elemento externo o bien integrando la agitación y la extracción en el mismo dispositivo.

La integración de la agitación y la extracción en el mismo dispositivo ha estado en continua evolución desde que en 1999 surgió la microextracción en barrita agitada (*stir bar sorptive extraction*, SBSE) [1]. La evolución de la SBSE está orientada al desarrollo de nuevas fases extractivas y al diseño de nuevas configuraciones tanto en el contexto de la microextracción en fase sólida como en fase líquida. En la Memoria se han realizado aportaciones en este ámbito.

## V.1. UNIDADES DE EXTRACCIÓN/AGITACIÓN BASADAS EN DISCOS

Las metodologías analíticas propuestas basadas en la utilización de unidades de extracción con agitación integrada en formato de disco se recuerdan en la **Figura 1**. Esta primera clasificación, se ha dividido a su vez en dos categorías: las que utilizan fritas de polietileno y las que emplean discos de borosilicato.

Las fritas de polietileno se utilizan como material sorbente, dando lugar a la técnica de microextracción en frita agitada. Por otro lado, los discos de borosilicato sirven como soporte y se han derivatizado con materiales nanoestructurados (nanocuernos de carbono oxidados) y con grupos octadecilo para estabilizar a un disolvente orgánico, dando lugar a la modalidad de microextracción en fase sólida y en fase líquida, respectivamente.

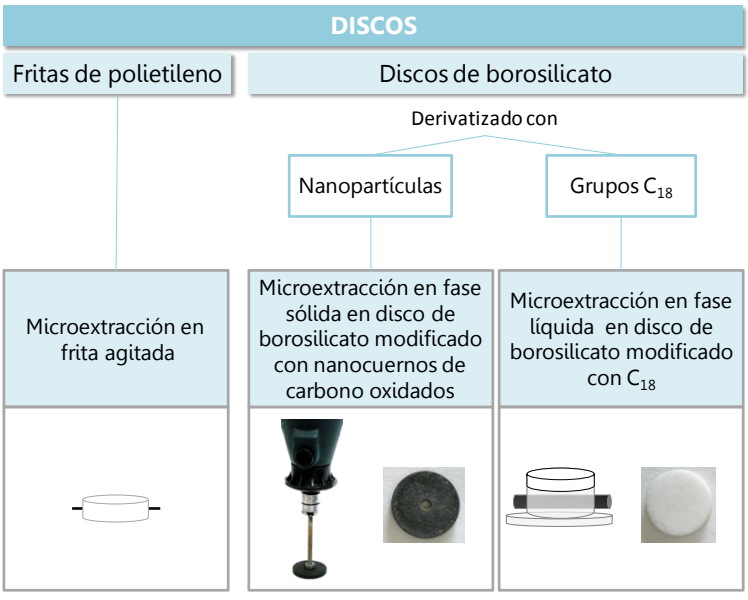


**Figura 1.** Unidades de extracción/agitación con formato de disco.

## V.1.1. FRITAS DE POLIETILENO COMO SORBENTES EN MICROEXTRACCIÓN EN FASE SÓLIDA

Las fritas se utilizan habitualmente en los cartuchos de extracción en fase sólida (SPE) para retener al sorbente o como medio para llevar a cabo procesos de filtración. Podemos encontrar: fritas de polietileno (PE) hidrofóbicas, de polietileno hidrofílicas, de politetrafluoroetileno (PTFE) y de polipropileno (PP). Las fritas de polietileno de naturaleza hidrofóbica se utilizan en la mayoría de cartuchos comerciales de SPE, y tienen un tamaño de poro de 20 µm. Estas fritas son muy similares a las de PTFE pero suponen una opción mucho más económica. Hasta la fecha, las fritas de PE se han utilizado como soporte para inmovilizar en sus poros materiales sorbentes, como materiales monolíticos [2] y polímeros de impresión molecular [3].

La investigación realizada en el **Capítulo 1** de la Memoria, consistió en la evaluación de fritas de PE hidrofóbicas como material sorbente polimérico en procesos de microextracción en fase sólida. Con este objetivo, la frita se perforó con un pequeño alambre de hierro, para hacer posible su agitación magnética dentro de la muestra. Dado el carácter hidrofóbico que presenta la frita de PE, este material está especialmente indicado para llevar a cabo el aislamiento y preconcentración de compuestos no polares. Por ello, los analitos propuestos como modelo son compuestos orgánicos volátiles (benceno, tolueno, etilbenceno, isómeros del xileno y estireno).

Los aspectos más relevantes de la etapa de microextracción y desorción se comentan a continuación (ver **Figura 2**). En cuanto al proceso de extracción, la primera variable que se evaluó fue el uso de un disolvente orgánico para acondicionar la frita. Si bien es cierto que esta etapa de activación o acondicionamiento es de especial interés, principalmente cuando se trabaja con fases ligadas a la sílice (como por ejemplo $C_{18}$), se observó que también en este caso era necesaria. Las señales analíticas se duplicaron o triplicaron (dependiendo del analito), cuando la frita se impregnaba en metanol (55 µL) antes de llevar

a cabo la extracción. Se comprobó por tanto que el acondicionamiento del sólido empleando un disolvente orgánico permite una adecuada interacción entre el analito y el sorbente.

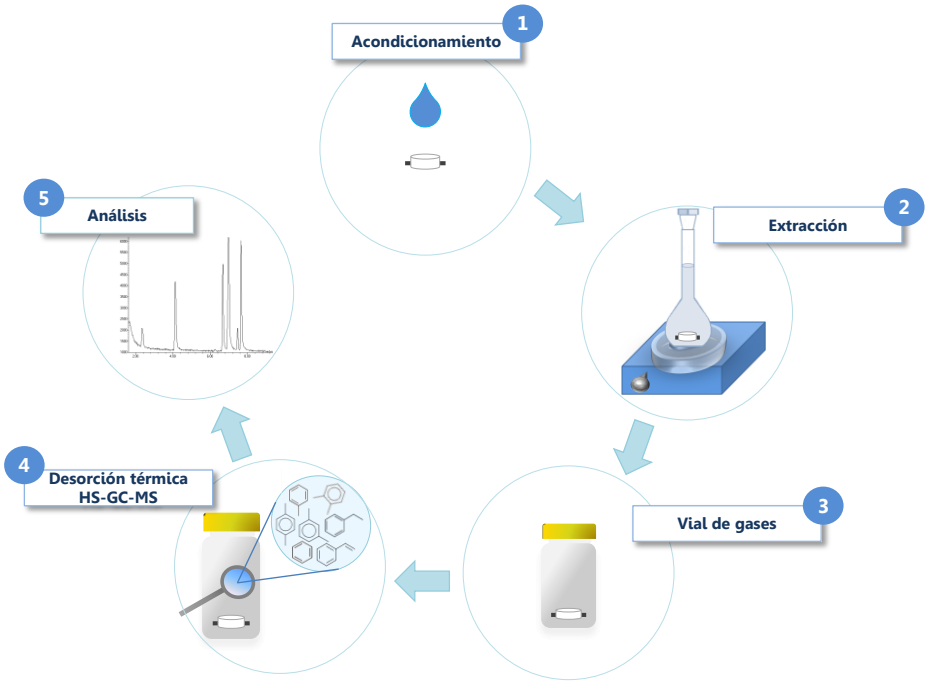


**Figura 2**. Procedimiento propuesto para la microextracción en frita agitada de compuestos orgánicos volátiles.

En el siguiente paso, la frita acondicionada se introdujo en la muestra y se agitó magnéticamente a 900 rpm durante 30 min. Se comprobó que, efectivamente, con la agitación magnética se obtenía una mejor eficiencia en la extracción, comparado frente a otros modos de agitación como mecánico o ultrasonidos. La cinética de la extracción es un aspecto clave a considerar cuando se estudia un proceso de extracción, y la máxima extracción se consigue cuando se alcanza el equilibrio de partición entre las fases implicadas. Este equilibrio puede desplazarse seleccionando adecuadamente algunas

variables químicas como la fuerza iónica. En este caso, la adición de un electrolito (NaCl) a la fase acuosa, favorece la extracción de los analitos diana mediante el efecto *salting-out*. El volumen de muestra influye positivamente en la sensibilidad de la metodología desarrollada ya que al aumentar el volumen aumenta también la cantidad de analito puesto en juego. Sin embargo, lo que sucede en la metodología desarrollada es que el dispositivo propuesto tiene una capacidad de agitación limitada, por lo tanto, aunque se aumente el volumen de muestra la frita no es capaz de homogeneizar todo el volumen.

En la última etapa, tiene lugar la desorción térmica de los analitos retenidos en la frita. Generalmente, la desorción térmica ofrece un aumento indirecto de la sensibilidad frente a la elución química ya que la totalidad de analito retenido se introduce en el sistema cromatográfico, mientras que en la elución química se inyecta una fracción, generalmente 2 µL del volumen de elución. Sin embargo, en la aplicación desarrollada, una vez finalizada la extracción, la frita se coloca en un vial de gases de 10 mL herméticamente cerrado para desorber térmicamente los analitos. Para ello, se calienta a 90ºC durante 30 min a 750 rpm y los analitos pasan de la frita al espacio de cabeza del vial, inyectándose 0.5 mL en el GC-MS, con la consiguiente dilución de los analitos. Dos posibles estrategias para mejorar la sensibilidad sería diseñar unidades específicas para desorber la frita en línea o elución química.

Con todo lo anteriormente descrito, se puede concluir que la evaluación de fritas como material sorbente en microextracción en fase sólida fue satisfactoria y que el acoplamiento con la cromatografía de gases mediante desorción térmica permitió alcanzar unos límites de detección entre 18 y 65 ng/L. La repetitividad osciló entre 3.8-8.2% y la reproducibilidad entre 6.4-14.8%. Se analizaron muestras de agua pero en ninguna de ellas se detectaron los analitos por lo que se fortificaron a una concentración de 500 ng/L, obteniéndose recuperaciones en torno al 94%.

Una de las limitaciones de la metodología propuesta es la reducida variedad de materiales disponibles, lo que hace que esté circunscrita a compuestos de naturaleza no polar. Las posibilidades de derivatización de la frita son escasas ya que es un material termolábil e inerte en el que es difícil introducir grupos funcionales.

## V.1.2. DISCOS DE BOROSILICATO COMO HERRAMIENTAS EN PROCESOS DE MICROEXTRACCIÓN

En esta Tesis Doctoral se propone el uso de discos de borosilicato como herramientas para aplicaciones analíticas. Se encuentran disponibles en el mercado en un amplio abanico de porosidades entre 1 y 500 µm, con diámetros comprendidos entre 5-380 mm.

Estos materiales presentan una elevada área superficial, fácil derivatización, así como unas propiedades químicas y mecánicas muy atractivas. Mediante la activación de la superficie inerte del disco, pueden crearse grupos hidroxilo en su superficie [4]. Una vez activados, pueden ser funcionalizados y servir como punto de anclaje de diferentes moléculas orgánicas en múltiples aplicaciones. En la Memoria se han hecho uso de discos de porosidad 10-16 µm y 16-40 µm, ambos de 20 mm de diámetro y se han funcionalizado con nanopartículas (**Capítulo 2**) y con grupos octadecilo (**Capítulo 6**).

### V.1.2.1. Discos de borosilicato modificados con nanopartículas

La Nanociencia y la Nanotecnología puede aplicarse al ámbito químico-analítico desde varias perspectivas, todas ellas complementarias y buscando dos objetivos básicos: miniaturizar los sistemas analíticos y explotar las excepcionales propiedades de la materia asociada a su tamaño nanométrico. Probablemente, han sido los nanotubos de carbono las nanopartículas base para las propuestas nanotecnológicas debido a sus excepcionales propiedades físico-químicas. Pero desde su descubrimiento, han sido muchos los esfuerzos investigadores orientados a la síntesis y caracterización de nuevas nanopartículas de

carbono. Entre ellas, los denominados nanocuernos de carbono monocapa (*single-walled carbon nanohorns*, SWNHs) se han revelado como un nanomaterial muy atractivo en nanotecnología debido a su potencial aplicabilidad en el diseño de electrodos, medios de almacenamiento de hidrógeno y metano y lubricantes sólidos. Por otro lado, estas nanopartículas, en base a su tamaño, forma y composición, resultan ser un candidato muy atractivo para su uso en el ámbito de la Química Analítica [5, 6].

La principal limitación que existe en el empleo de las nanopartículas de carbono en microextracción en fase sólida es la tendencia a la agregación que presentan. La agregación del sorbente es un inconveniente para la retención de los analitos ya que disminuye la superficie activa disponible para interaccionar con los compuestos de interés. Por ello, su uso empaquetadas en cartuchos de extracción en fase sólida no resulta especialmente adecuado.

Sin embargo, las nanopartículas de carbono resultan especialmente competitivas en las técnicas de extracción miniaturizadas [7]. La microextracción en fase sólida emplea pequeñas cantidades de sorbente, en el orden de los microgramos, para aislar los analitos de interés de la muestra. Para mantener su eficacia, el material sorbente debe tener una gran capacidad de adsorción, de manera que se puedan alcanzar elevados factores de enriquecimiento y por tanto alcanzar valores de sensibilidad suficientemente elevados para poder detectar los analitos a bajos niveles de concentración, en la mayoría de los casos, fijados por la legislación.

En el **Capítulo 2** de esta Memoria se utilizan nanocuernos de carbono oxidados (o-SWNHs) inmovilizados sobre discos de borosilicato para la determinación de benzofenona-3 en muestras de agua de piscina. El proceso de síntesis del disco funcionalizado consta de tres etapas. En primer lugar, es necesario llevar a cabo la activación del disco para la creación de grupos hidroxilos superficiales mediante un tratamiento con ácidos y bases fuertes. A continuación, el disco se lava y se seca, y se funcionaliza con grupos amino introduciéndolo

en una disolución de 3-aminopropiltrietoxilano (APTS). Se retira el exceso de APTS y se introduce en una disolución que contiene glutaraldehído (GA).

Por otro lado, se oxidan los SWNHs, para lo cual se tomaron las condiciones descritas por Yoshida y Sano [8] que implican una irradiación del sólido durante 10 min en un horno de microondas a 800 W de potencia. De los distintos procedimientos descritos en bibliografía, se optó por la energía de microondas debido a la mayor rapidez del mismo frente al empleo de radiación UV. Conviene resaltar que la facilidad de oxidación de los SWNHs contrasta notablemente con la dificultad de llevar a cabo el mismo procedimiento por ejemplo con nanotubos de carbono, ya que se requeriría tratamiento con ácidos fuertes y varias etapas, dificultando la obtención de nanotubos oxidados con un porcentaje de grupos funcionales reproducible.

Una vez oxidados, se dispersaron en agua mediante la aplicación de energía de ultrasonidos durante 60 min y se centrifugaron. De esta manera, se consiguen eliminar los nanocuernos que no se hayan oxidado. En la tercera etapa tiene lugar la inmovilización de los o-SWNHs sobre el soporte. Para ello, se introduce el disco activado en una disolución de dimetilformamida que contiene a los nanocuernos oxidados y carbodiimida, manteniendo el sistema en atmósfera inerte. Los discos obtenidos se caracterizaron mediante SEM, observándose formaciones rugosas que se corresponden con la presencia de dalhias (agregados estables de o-SWNHs).

En cuanto al dispositivo de extracción/agitación, el disco sintetizado se acopló a un taladro portátil, y se estudiaron todas las variables que influyen en el proceso de extracción, tales como la fuerza iónica, el volumen de muestra, la profundidad a la que se introduce el dispositivo en la muestra y el tiempo de extracción. En cuanto al volumen de muestra es importante resaltar que se observó que la unidad de extracción era capaz de homogeneizar hasta 200 mL de muestra, y que para volúmenes superiores la señal analítica era independiente del volumen de muestra, lo cual pone de manifiesto la posibilidad de emplear la herramienta desarrollada para llevar a cabo extracciones *in situ*.

Para llevar a cabo la elución, se acopló el disco al sistema de vacío de SPE y se pasaron 5 mL de acetona a su través. Los extractos se evaporaron y redisolvieron en 100 µL de metanol, y se inyectaron en el UPLC-DAD. El límite de detección del método fue de 0.16 µg/L, alcanzándose factores de preconcentración de 1379, y una recuperación absoluta del orden del 70%. Se evaluó la precisión para un mismo disco a dos niveles de concentración y también se estudió la precisión entre diferentes síntesis, siendo en este caso inferior al 11.8%. En cuanto a la aplicación de la metodología desarrollada en aguas de piscina, no se encontraron muestras positivas, así que se fortificaron con el analito a una concentración de 5 µg/L y las recuperaciones estuvieron comprendidas entre 78 y 110%.

La principal ventaja que ofrece el acoplamiento del disco con el sólido sorbente inmovilizado al taladro es la posibilidad de llevar a cabo extracciones *in situ*, lo cual resulta del todo inviable tanto si se inmovilizan las nanopartículas sobre fibras como en membranas. Por otro lado, la capacidad de extracción de fibras modificadas con nanocuernos oxidados es menor ya que la cantidad de sorbente que se puede inmovilizar/retener en los poros de la fibra es inferior [9].

### V.1.2.2. Discos de borosilicato modificados con grupos octadecilo

En el **Capítulo 6**, se utilizó el disco de borosilicato en la modalidad de microextracción en fase líquida. Para ello, se derivatizó el disco con cadenas de $C_{18}$, capaces de estabilizar al disolvente orgánico mediante interacciones hidrofóbicas.

Está descrito en bibliografía que este proceso puede realizarse mediante la tecnología sol-gel o bien mediante métodos *grafting*. La reacción sol-gel, que se realiza generalmente en etanol, da lugar a un recubrimiento eficiente, con una conformación cristalina. El método *grafting* se lleva a cabo en tolueno y da como resultado una cobertura inferior. Sin embargo, este revestimiento presenta una conformación diferente que favorece la estabilización de un disolvente en la superficie del disco. Por ello tanto, se seleccionó este

último enfoque. Finalmente, el disco se lava varias veces en abundante agua y se seca antes de su uso.

La síntesis del disco consta tres etapas. El primer paso consiste en la activación del disco. Para ello, se introduce en una mezcla de ácido sulfúrico: peróxido de hidrógeno (2:1 v/v) a 100ºC durante 20 min. El disco se lava para neutralizar el pH y se seca en un horno a 80ºC durante 12 h. En una segunda etapa, el disco activado, se introduce en 50 mL de tolueno conteniendo 0.5 mL de trimetoxi(octadecil)silano, durante 12 h a 120 ºC.

El disco resultante se caracterizó mediante un rápido ensayo colorimétrico con fenolftaleína que confirmó que la derivatización se había llevado a cabo. Se realizó también un espectro de IR, observándose unas bandas de baja intensidad a 2900-300 $cm^{-1}$, las cuales representan las vibraciones C-H de la fase octadecil. Es precisamente esta baja intensidad la que explicaría que en este caso la microextracción tiene lugar en fase líquida ya que el recubrimiento con $C_{18}$ es suficiente para estabilizar al disolvente orgánico no polar pero no tan elevado como para ser el mismo el responsable de la extracción. Por lo tanto, para utilizar el $C_{18}$ bajo el formato de extracción en fase sólida debería mejorarse el recubrimiento.

Para llevar a cabo la agitación del disco sintetizado, se diseñó una unidad a partir de una jeringa del 20 mL. Se trata de una estructura de plástico del mismo diámetro que el disco, perforada con un imán magnético, que hace posible su agitación.

Los analitos objeto de estudio fueron nueve herbicidas, pertenecientes a la familia de las triazinas (simazina, simetrin, atrazina, secbumetón, prometón, terbumetón, propazina, prometrin y terbutrin) que son empleados de forma extensiva para controlar las malas hierbas. Estos herbicidas se aplican directamente sobre el suelo con lo cual puede generar lixiviaciones y contaminar las aguas subterráneas y superficiales.

Los aspectos más relevantes de la etapa de microextracción y elución se comentan a continuación. En primer lugar, se evaluó el comportamiento del disco sin modificar, el disco modificado con $C_{18}$, y el disco con distintos disolventes estabilizados sobre el $C_{18}$ (se ensayaron: hexano, cloroforno, acetato de etilo, tolueno y metanol). A la luz de los resultados obtenidos, las mejores eficiencias en la extracción se obtuvieron cuando se utilizó el tolueno soportado (responsable de la extracción). La elevada porosidad del disco tiene un doble efecto; por un lado permite emplear volúmenes relativamente grandes (especialmente si se compara con otras técnicas de microextracción) de disolvente orgánico, del orden de los 300 µL; por otro lado, la superficie de contacto entre el disolvente y la muestra es mayor.

A continuación, se prosiguió estudiando las variables que influyen en la eficiencia de la extracción. Se evaluó de manera conjunta la velocidad de agitación (hasta 1400 rpm) y el volumen de muestra (200, 500 y 750 mL), observándose que, a menor volumen de muestra, la mejor eficiencia en la extracción se obtiene a velocidades de agitación intermedia. Lo que sucede en este caso es que si se aumenta la velocidad de agitación, el vórtice creado justo encima de la unidad de extracción hace que esté en contacto con el aire y no con la muestra. Cuando se emplean volúmenes de 500 mL, la extracción aumenta al incrementar la velocidad de agitación. Sin embargo cuando se emplean volúmenes de 750 mL, no se observa incremento en la señal si se comparan velocidades de agitación medias y altas ya que la unidad de extracción/agitación no es capaz de movilizar/homogeneizar toda la muestra. Finalmente, se estudió el tiempo de extracción, y se observó que por encima de 30 min, las extracciones empezaban a ser irreproducibles, hecho que se atribuyó a solubilización parcial del tolueno en la muestra, ya que su solubilidad en agua es de 0.52 g/L.

Se realizó la elución química de las triazinas extraídas acoplando el disco al sistema de vacío de SPE, y se pasó 1 mL de metanol a su través. Los extractos se evaporaron con dos finalidades, por un lado para hacerlos compatibles con la instrumentación empleada, ya

que el tolueno es inmiscible con la fase móvil del sistema cromatográfico y por otro lado para mejorar la sensibilidad de las determinaciones. Finalmente, se redisolvieron en 100 µL de metanol y se analizaron mediante UPLC-DAD. Los límites de detección del método estuvieron comprendidos en el intervalo 0.14-0.56 µg/L, con precisión mejor del 7.3%. Los factores de preconcentración, estuvieron comprendidos entre 79-839; teniendo en cuenta que el factor máximo de preconcentración es de 5000, las recuperaciones absolutas obtenidas estarían comprendidas entre el 1.6 y el 16.8%.

Es interesante destacar la relación observada entre el log P de los analitos y los factores de preconcentración obtenidos. Así, los analitos más hidrofóbicos fueron los que mejor se extrajeron. En cuanto a la aplicabilidad en muestras ambientales, se realizó un estudio de recuperación fortificando la muestra a una concentración de 3 µg/L ya que no se encontraron muestras positivas, y las recuperaciones relativas estuvieron comprendidas entre el 70 y 120%.

## V.1.3. EVALUACIÓN CONJUNTA DE LAS METODOLOGÍAS DESARROLLADAS BASADAS EN EL EMPLEO DE DISCOS

En la **Tabla 1** se recogen a modo de resumen los parámetros y los resultados más destacables de las metodologías propuestas basadas en el uso de discos, como medio extractante (**Capítulo 1**) o bien mediante el empleo de discos de borosilicato como herramienta para aplicaciones analíticas (**Capítulos 2** y **6**).

**Tabla 1**. Comparación de las metodologías desarrolladas en los Capítulos 1, 2 y 6.

| | **Capítulo 1** | **Capítulo 2** | **Capítulo 6** |
|---|---|---|---|
| Muestra | Aguas ambientales | Agua de piscina | Aguas ambientales |
| Analitos | Compuestos orgánicos volátiles | Benzofenona-3 | Triazinas |
| Modalidad | µ-SPE | µ-SPE | LPME |
| Porosidad (µm) | 20 | 16-40 | 10-16 |
| Derivatización | No | o-SWNHs | $C_{18}$ con tolueno estabilizado |
| Caracterización | No | SEM | IR |
| Fuerza iónica (g/L NaCl) | 100 | 0 | 50 |
| Volumen de muestra (mL) | 100 | 200 | 500 |
| Tiempo de extracción (min) | 30 | 15 | 30 |
| Agitación (rpm) | 900 | 3000 | 1400 |
| Elución | Desorción térmica | Elución química (acetona) | Elución química (metanol) |
| Instrumentación | GC-MS | UPLC-DAD | UPLC-DAD |
| LD (ng/mL) | 0.018-0.065 | 0.16 | 0.14-0.56 |
| DER (%) | <8.2 | 11.9 | <7.3 |
| Factor de preconcentración | 157-998 | 1379 | 79-839 |
| Recuperación absoluta (%) | 8.7-55.4 | 68.9 | 1.6-16.8 |
| Recuperación relativa (%) | 73-121 | 80-110 | 70-120 |

Por último, en la **Tabla 2** se realiza un análisis DAFO (debilidades, amenazas, fortalezas y oportunidades) de cada metodología.

**Tabla 2**. Análisis DAFO de las metodologías desarrolladas en los Capítulos 1, 2 y 6.

| | **D**ebilidades | **A**menazas | **F**ortalezas | **O**portunidades |
|---|---|---|---|---|
| **Capítulo 1** | Limitada a su aplicación a compuestos no polares<br><br>Limitada la temperatura de desorción que puede emplearse | Dificultad de automatización<br><br>Limitada la derivatización de la frita | Altos factores de preconcentración<br><br>Económico | Diseñar interfase para desorción térmica en línea<br><br>Compatible con LC si se realiza elución química |
| **Capítulo 2** | Síntesis tediosa<br><br>Dificultad de saber exactamente la cantidad de nanopartículas inmovilizadas | Dificultad de automatización | Altos factores de preconcentración<br><br>Elevada capacidad de agitación<br><br>Capaz de homogeneizar a un volumen elevado de muestra | Potencial para ser utilizada en extracciones *in situ*<br><br>Podría derivatizarse con otras nanopartículas |
| **Capítulo 6** | Bajas recuperaciones absolutas<br><br>Parcial solubilización del extractante en la muestra | Dificultad de automatización | Altos factores de preconcentración<br><br>Capaz de homogeneizar a un volumen elevado de muestra | Podrían estabilizarse otros disolventes orgánicos cambiando la derivatización<br><br>Potencial de ser utilizado en la modalidad de µSPE si se aumenta la cantidad de fase activa |

## V.2. UNIDADES DE EXTRACCIÓN/AGITACIÓN BASADAS EN EL EMPLEO DE MEMBRANAS

Las metodologías analíticas propuestas basadas en la utilización de unidades de extracción con agitación integrada en las cuales se emplean membranas, se resumen en la **Figura 3**. Se ha dividido a su vez en dos categorías, atendiendo al rol que desempeña la membrana dentro del sistema, ya que pueden actuar como separación entre las distintas fases implicadas o bien ser el medio sorbente responsable de la extracción.

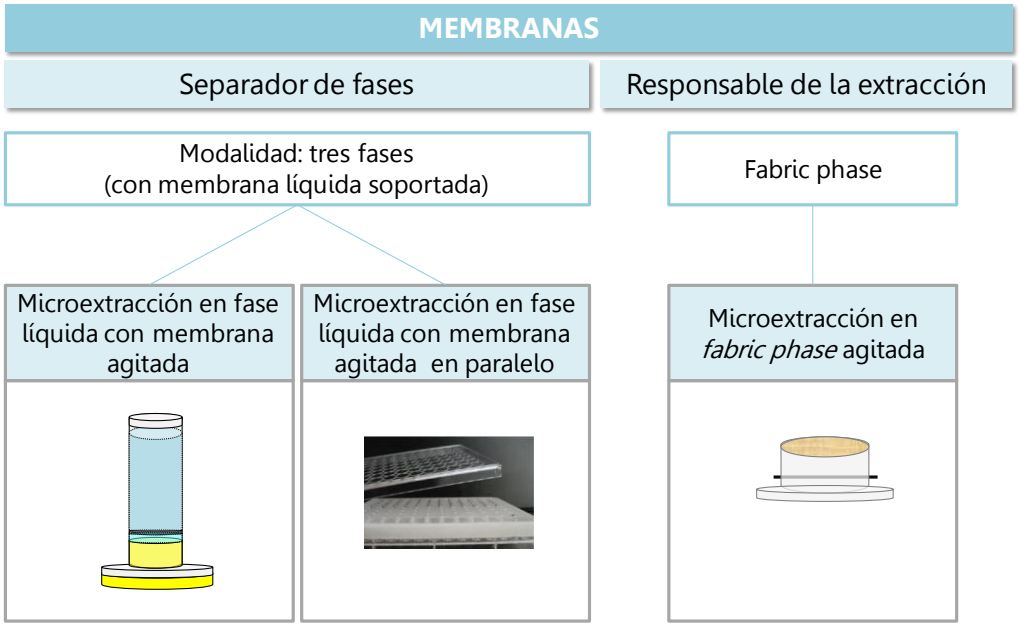


**Figura 3.** Unidades de extracción/agitación que emplean membranas.

## V.2.1. MEMBRANAS COMO SEPARADORAS DE FASES

Tradicionalmente, las membranas se han empleado en diferentes procesos de separación, como por ejemplo, la desalinización, la diálisis, la ultrafiltración, la separación de gases, la deshumidificación, la ósmosis inversa y la electrodiálisis. Las membranas actúan como un separador de fases, controlando la transferencia de masa entre ellas y evitando los problemas asociados a la formación de emulsiones. Además, la microextracción en fase líquida basada en membranas se utiliza habitualmente en la preparación de muestras biológicas ya que evita la presencia de biomoléculas en el extracto final, haciendo que sea directamente compatible con LC. La gran variedad de membranas disponibles comercialmente de diferente composición, capacidad, morfología y formatos (planas o fibra hueca) hace que sean herramientas muy versátiles.

En este contexto surge la técnica de microextracción con membrana líquida soportada (*supported liquid membrane extraction*, SLME), propuesta por Jönsson en 1992 [10], en la cual el disolvente orgánico se dispone formando una película delgada sobre una membrana de teflón. Posteriormente, las membranas de polipropileno con formato de fibra hueca han dado lugar al desarrollo de la técnica de microextracción en fibra hueca (*hollow fiber membrane liquid phase microextraction*, HF-LPME) [11-13], propuesta en 1999 por el grupo de los profesores Pedersen-Bjergaard y Rasmussen. En esencia, consiste en impregnar el interior y los poros de una fibra hueca con el disolvente orgánico (modalidad de dos fases) o bien disponer una fase acuosa en el lumen de la fibra hueca de manera que el medio orgánico se localice exclusivamente en los poros de la fibra, separando las dos fases acuosas (modalidad de tres fases). La modalidad de dos fases está indicada para la extracción de compuestos hidrofóbicos, mientras que la modalidad de tres fases tiene mayor aplicación para compuestos hidrofóbicos ionizables.

De entre los diferentes sistemas de extracción basados en el uso de membranas existentes [14], en la presente Memoria se ha trabajado con sistemas de extracción en tres fases con membranas líquidas soportadas (*supported liquid membrane*, SLM), destinadas a la

determinación de fármacos en biofluidos (**Capítulos 7** y **8**). En esta modalidad, el paso de los analitos tiene lugar mediante una difusión pasiva y el gradiente de pH establecido a ambos lados de la membrana es la fuerza impulsora de la extracción. Esta modalidad se emplea cuando se pretende extraer analitos básicos o ácidos con grupos ionizables; de esta forma el analito se encuentra en la forma neutra en la fase donadora, e ionizado en la fase aceptora. Dependiendo de la naturaleza de los compuestos, se pueden establecer diferentes gradientes de pH. Para la extracción de analitos básicos, el pH de la muestra se debe ajustar hasta basicidad mientras que el de la fase aceptora debe ser ácido. Por el contrario, en el caso de analitos ácidos, se acidifican las muestras y se seleccionan fases aceptoras alcalinas. La transferencia de masa del analito desde la fase acuosa donadora a la fase acuosa aceptora continúa hasta que se alcanza el equilibrio termodinámico o se interrumpe la extracción.

#### V.2.1.1. Microextracción en fase líquida usando membranas líquidas soportadas

##### V.2.1.1.1. Microextracción en fase líquida con membrana agitada para muestras de disponibilidad limitada

El primer sistema de extracción propuesto, descrito en el **Capítulo 7**, está inspirado en la técnica de microextracción con membrana líquida agitada (*stir membrane liquid-liquid-liquid microextraction*, SM-LLLME) [15]. Tradicionalmente, el uso de esta técnica estaba orientado al análisis de aguas de interés ambiental, ya que se necesitaban al menos 10-15 mL de muestra para llevar a cabo la extracción. Con el objetivo de extender su aplicabilidad al bioanálisis, se modificó el diseño de la unidad de extracción original con vistas a reducir el volumen de muestra a unos pocos mililitros, adaptándola al análisis de muestras de disponibilidad limitada.

La unidad de extracción está constituida por cuatro elementos básicos (ver **Figura 4**). El proceso de ensamblaje es sencillo. En primer lugar se encajan el tapón (i) y el elemento interno (ii). La fijación de los elementos (ii) y (iv) es crucial, ya que definen la cámara

interna donde más tarde se situará el extractante. Una vez añadido éste, se deposita la membrana (iii) sobre la unidad, fijándola por desplazamiento del elemento externo (iv). La modificación propuesta consiste en incrementar la altura esta última pieza, generando así una cámara externa sobre la membrana que alberga la muestra.

Este sistema se utilizó para llevar a cabo la determinación de paracetamol en muestras de saliva. Para ello, el paracetamol se extrae desde la muestra de saliva (2 mL), a través de la membrana impregnada con 1-octanol hasta la fase aceptora (50 µL de disolución NaOH 10 mM), que se inyecta directamente en el HPLC-UV.

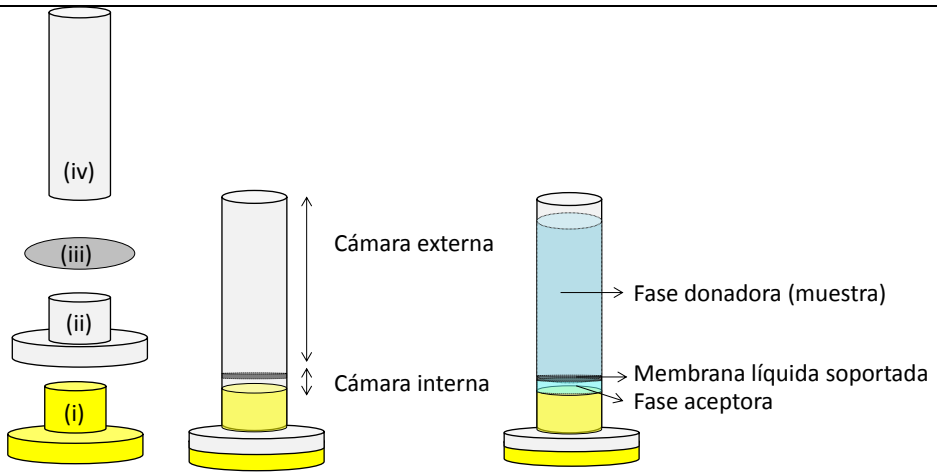


**Figura 4.** Elementos básicos de la unidad de microextracción con membrana agitada. Más detalles en el texto.

En los procesos de microextracción en fase líquida de tres fases, es clave la correcta selección del disolvente orgánico utilizado para formar la SLM así como el tipo de membrana empleada. A continuación, se comentan los requisitos que deben cumplir.

*Selección del disolvente orgánico para formar la membrana líquida soportada.* Los disolventes orgánicos empleados para la formación de la SLM deben de presentar una serie

de características, de entre las que destacan: baja volatilidad; inmiscibilidad en agua; adecuada viscosidad, para quedar fuertemente retenidos en los poros de la membrana pero permitir la difusión de analitos a su través; y finalmente deben también presentar coeficientes de partición para los analitos que se van a extraer suficientemente elevados como para que la extracción sea eficiente.

El carácter volátil del disolvente se relaciona con la facilidad que tienen para evaporarse. Esto es importante porque, si bien es cierto que las membranas normalmente se preparan inmediatamente antes de realizar las extracciones, estarán durante un breve periodo de tiempo en contacto con el aire (generalmente menos de 2 min) hasta que se pongan en contacto con la muestra. Por ello, ha de tenerse en cuenta el punto de ebullición de dicho disolvente. Una vez que la SLM se introduzca en la muestra, los problemas asociados a pérdidas del disolvente por evaporación desaparecen ya que queda protegida por la muestra y además el sistema está cerrado. Los disolventes que presenten una volatilidad elevada, como el tolueno o el 1-cloropentano, no son adecuados para formar la SLM ya que al evaporarse de forma rápida, daría lugar a SLM muy inestables. Los disolventes más utilizados descritos en la bibliografía para la formación de la SLM son el dihexileter y el 1-octanol. Ambos se caracterizan por presentar puntos de ebullición elevados, por encima de 195 ºC. Se recomienda por tanto, como norma general, no utilizar disolventes que presenten puntos de ebullición por debajo de los 190-200 ºC.

Además de la baja volatilidad, otro requisito fundamental que deben de cumplir los disolventes es ser inmiscibles con agua. Las membranas formadas a partir de dihexileter serán muy estables ya que este presenta baja solubilidad en agua (<110 µg/mL). Sin embargo, el 1-octanol presenta elevada solubilidad en agua (1200 µg/mL) y por ello este disolvente se disuelve parcialmente en la muestra o en la fase aceptora durante la extracción. Teóricamente, aproximadamente un 11% del 1-octanol que forma una SLM puede perderse en 1 mL de muestra. Esto se ha demostrado experimentalmente por el grupo del Prof. Perdersen-Bjergaard. Para ello, se realizaron extracciones con HF-LPME y

analizaron las muestras mediante GC-MS, encontrándose trazas de 1-octanol. Se recomienda, por tanto, no utilizar disolventes con solubilidad en agua que exceda de 200-400 µg/mL para evitar pérdidas del disolvente.

A pesar de este último inconveniente, en el **Capítulo 7** de la Memoria se utilizó 1-octanol como disolvente para formar la SLM en la SM-LLLME ya que está ampliamente descrito su uso en bibliografía y el trabajo estaba orientado al diseño de una configuración compatible con muestras de disponibilidad limitada. En la investigación recogida en el **Capítulo 8**, en la que se utilizaron también membranas líquida soportadas, se realizó un estudio más exhaustivo del disolvente empleado para formar la SLM.

*Selección de la membrana.* Los criterios tenidos en cuenta a la hora de seleccionar la membrana fueron: (i) la naturaleza y la forma mediante la cual va a ser fijada, y (ii) el espesor. La forma en la que se fija la membrana en la unidad de extracción determina la naturaleza de la membrana que puede emplearse. Como se ha descrito con anterioridad, en esta aplicación la membrana se fija a la unidad de extracción por desplazamiento del elemento externo sobre el interno, por lo tanto, es imprescindible que se empleen membranas flexibles y delgadas para evitar roturas. La cinta de teflón de 100 µm de espesor resultó ser la mejor candidata dado su carácter elástico.

En lo que respecta a las características analíticas del método, se obtuvieron factores de preconcentración de 10 (siendo el máximo teórico 40, ya que se pasa de un volumen de muestra de 2 mL a 50 µL de fase aceptora), lo que significa que la recuperación absoluta está en torno al 25%. En aquel momento, el bajo porcentaje de recuperación se atribuyó al hecho que las técnicas de microextracción son procedimientos de extracción no exhaustivos y por ende es complicado obtener un alto rendimiento. Sin embargo, este hecho podría ser también explicado atendiendo al log P (siendo P el coeficiente de partición octanol/agua). El log P del paracetamol es de 0.46, y está descrito en la bibliografía que las máximas recuperaciones en la extracción se obtienen cuando el log P

del analito se encuentra comprendido entre 2 y 4 [16]. El límite de detección del método fue de 0.5 µg/L, la precisión fue mejor del 7% y la recuperación estuvo alrededor del 93%.

Se realizó también un estudio farmacocinético para observar la evolución de la concentración de paracetamol en la saliva con el tiempo. Para ello, se tomaron muestras periódicas de saliva tras la ingesta del fármaco. Se observó que la concentración en saliva aumentaba hasta los 30-60 min, y después disminuía progresivamente.

#### V.2.1.1.2. Microextracción en fase líquida con membrana en paralelo

La principal limitación que presenta SM-LLLPME es el reducido número de muestras que pueden ser procesadas al mismo tiempo y la difícil automatización de la técnica. Estos dos aspectos hacen que su aplicación en el análisis de rutina sea un reto. Para tratar de solucionar estas limitaciones, se han desarrollado nuevos procedimientos de extracción LLE miniaturizados. En este contexto, la implementación de formatos multipocillo se ha llevado a cabo de forma satisfactoria en la técnica de extracción con electromembranas en paralelo (*parallel electromembrane extraction*, Pa-EME) [17], en HF-LPME en un nuevo formato de 96 [18] y en la extracción con membrana líquida en paralelo (*parallel artificial liquid membrane extraction*, PALME) [19].

En el **Capítulo 8** se utiliza PALME para la determinación de seis anti-inflamatorios en muestras de plasma. PALME está compuesto por cuatro elementos que pueden adquirirse de forma comercial: una bandeja de 96 pocillos (0.5 mL), tiras de 8 tubos de pared fina, membrana de polipropileno y una tapadera. Las muestras de plasma (250 µL) se pipetean sobre la bandeja 96 pocillos y se diluyen 1:1 (v/v) con HCl 125 mM. A continuación se impregna la membrana con el disolvente orgánico (2 µl de dihexileter) y se pipetéa la fase aceptora (50 µL de disolución amoniacal 25 mM). Se coloca encima una tapadera para evitar una posible evaporación del disolvente, así como para que no se den fenónemos de contaminación cruzada por salpicaduras. El sistema con formato sandwich se sitúa en una plataforma vibradora para que tenga lugar la extracción. Los analitos diana protonados, se

extraen desde la muestra, a través de la SLM hasta la fase aceptora. El extracto es analizado directamente mediante HPLC-DAD. El esquema del procedimiento seguido se esquematiza en las **Figuras 5** y **6**.

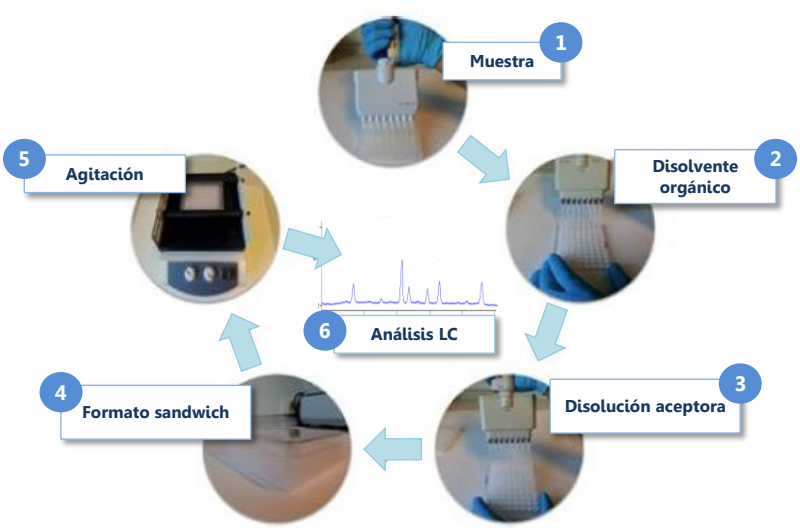


**Figura 5.** Procedimiento llevado a cabo en PALME. Adaptada de la referencia 20.

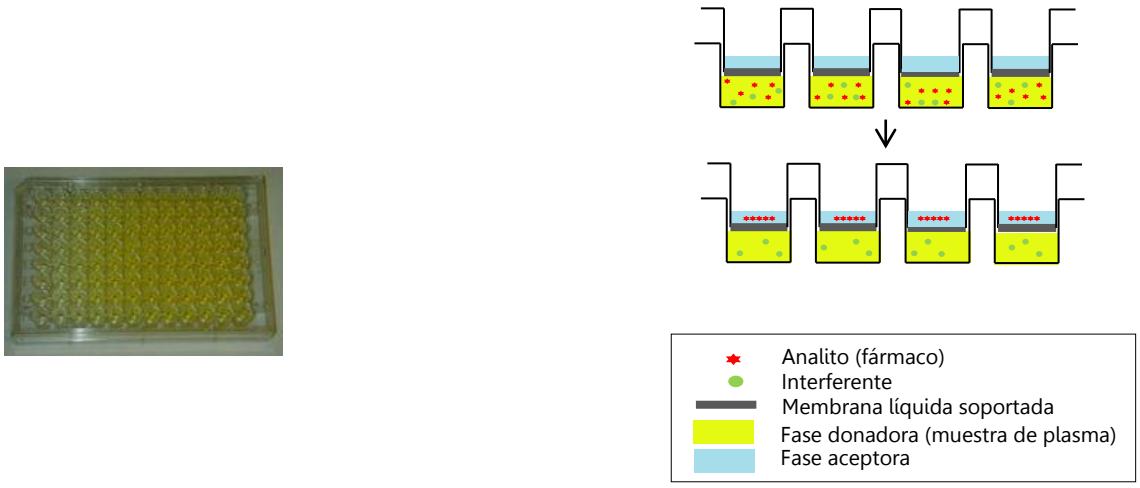


**Figura 6.** Fotografía de PALME y detalle de varios pocillos.

A continuación se comentan dos parámetros clave de la optimización llevada a cabo.

La *selección del disolvente orgánico para formar la membrana líquida soportada* fue una variable clave en este trabajo experimental. Se ensayaron nueve disolventes orgánicos de distinta naturaleza: 1-hexil-1-decanol, 1-nonanol, 1-octanol, 1-decanol, dodecil acetato, 2-nitrofenil octil eter, 2,2-dimetil-1-propil benceno, isopentil benceno, y dihexileter. Se comprobó que con tan sólo 2 µL de disolvente, la membrana de 6 mm de diámetro quedaba completamente impregnada. Las extracciones se realizaron por cuadruplicado, y dejando un tiempo relativamente largo (30 min). Se obtuvieron experimentalmente recuperaciones cercanas al 100% con tres de los disolventes evaluados: 2,2-dimetil-1-propil benceno, isopentil benceno, y dihexil eter.

En cuanto a la *membrana empleada*, a diferencia de la aplicación anterior, en este trabajo experimental no se fija por acción mecánica sino que se sella por calor con un soldador a 185 ºC durante 2 segundos. En principio, podría utilizarse cualquier tipo de membrana, ya que los problemas asociados a fracturas se evitan. Se han utilizado membranas de polipropileno (PP) tras ser comparadas con las de fluoruro de polidivinil (PVDF). Para llevar a cabo esta comparación, se realizaron dos calibrados con extracción (uno con cada tipo de membrana) en el intervalo de 0.25-10 ng/mL, observándose que con las de PP había buena linealidad ($R^2$=0.997-1.000), mientras que con las de PDVF no ($R^2$=0.9235-0.9407). Además, las extracciones realizadas con PVDF eran altamente irreproducibles (RSD: 20-55%). Esto es debido a interacción no específicas de los analitos con la membrana de PVDF, por lo tanto se optó finalmente por emplear las membranas de PP.

Con respecto a las características analíticas del método, los límites de detección estuvieron comprendidos entre 6.4 y 22.9 ng/mL y la precisión, en términos de repetitividad y reproducibilidad (expresada como desviación estándar relativa) fue del 15% y 22%, respectivamente. El estudio de la recuperación se realizó en muestras de plasma

fortificadas a tres niveles de concentración (50, 100, 500 ng/mL) y por triplicado, estando en todos los casos los valores comprendidos entre el 79 y el 130%.

La evolución de PALME va encaminada a la mejora del procedimiento de tratamiento de muestra, estando materializada en dos líneas de investigación complementarias:

*Automatización para su aplicación en los laboratorios de rutina*

Actualmente PALME se encuentra pendiente de patente (número US61/708 325). A pesar de que cómo se ha descrito anteriormente, las membranas se sellaban con un soldador de manera manual, se está trabajando en un dispositivo comercial con membranas de PP. Se trata por tanto de una metodología con gran potencial de ser automatizada en los laboratorios de rutina. Además, sería también posible su implementación en bandejas de 384.

*PALME para la extracción de compuestos polares*

Para que definitivamente PALME se consolide como una herramienta útil en el tratamiento de muestras, es necesario ampliar su aplicabilidad a otras matrices complejas (sangre, leche materna, orina o sudor), así como a otro tipo de compuestos. La extracción de compuestos no polares podría llevarse a cabo trabajando en la modalidad de dos fases, de tal manera que si se utilizara una fase aceptora orgánica, podría ser compatible con GC. Para la extracción de compuestos polares, es necesario utilizar moléculas transportadoras en el sistema. A continuación se va a comentar este último aspecto con más detalle.

Como se ha descrito anteriormente, la microextracción líquida en tres fases está indicada para analitos de naturaleza hidrofóbica (log P>2) que presenten en su estructura grupos ionizables. Los compuestos hidrofílicos, caracterizados por presentar log P<2, no pueden extraerse mediante la microextracción líquida-líquida-líquida convencional debido a la baja afinidad por el disolvente de la SLM. Para llevar a cabo la extracción de compuestos polares se requiere la adición de moléculas transportadoras (más conocidas como "*carriers*") a la

fase acuosa o a la fase donadora. Estas moléculas transportadoras son capaces de formar complejos hidrofóbicos con los analitos ionizados y así hacer posible su paso a través de la SLM hasta la fase aceptora.

Para demostrar el potencial de PALME con compuestos polares, se hicieron unos ensayos preliminares en las últimas semanas de la estancia predoctoral. Se escogieron cuatro compuestos polares de naturaleza básica: metaraminol, atenolol, salbutamol y practolol.

Las condiciones experimentales se resumen a continuación. Como fase donadora, se utilizó 0.5 mL de buffer fosfato 25 mM a pH 7, la fase aceptora fue 50 mM de ácido clorhídrico (50 µL) y se utilizó una membrana de PP impregnada con 1-octanol (2 µL) para formar la SLM. Basándonos en nuestra experiencia previa, el tiempo de extracción se fijó en 30 min, y la agitación se mantuvo a 900 rpm. La técnica instrumental utilizada fue HPLC-DAD.

Se utilizaron moléculas transportadoras de distinta naturaleza:

- Fosfato: tris (2-etilhexil) fosfato, tributilfosfato, di (2-etilhexilfosfato) y varias mezclas entre ellos.
- Sulfato: ácido 1-octano sulfónico y ácido 1-heptano sulfónico.
- Carboxílico: ácido octanoico.

Las moléculas transportadoras de naturaleza fosfato fueron añadidas a la membrana líquida soportada en un 15% (v/v) y los sulfatos y carboxílicos se añadieron a la muestra a una concentración de 25 mM. Los resultados preliminares se muestran a continuación en la **Tabla 3**. Se observan recuperaciones cercanas al 100% al mezclar di(2-etilhexil)fosfato y tributilfosfato en proporción 1:1 (v/v) y añadirlo a la SLM en un 15%. Estos resultados son prometedores y están en concordancia con los experimentos llevados a cabo en HF-LPME [21], augurando que los principios de la extracción con moléculas transportadoras son trasladables a PALME.

**Tabla 3**. Recuperaciones obtenidas en la extracción de compuestos polares con PALME.

| | Recuperación (%) (% DER) | | | |
|---|---|---|---|---|
| | **Metaraminol** | **Atenolol** | **Salbutamol** | **Practolol** |
| **AÑADIDO A LA SLM** | | | | |
| **Fosfatos** | | | | |
| Tris (2-etilhexil) fosfato | 26 (22) | - | 9 (28) | 12 (27) |
| Tributilfosfato | - | - | 1 (23) | 2 (28) |
| Di (2-etilhexil) fosfato | 61 (10) | 57 (11) | 72 (13) | 63 (27) |
| Di (2-etilhexil) fosfato + tris (2-etilhexil) fosfato (1:1, v/v) | 82 (4) | 53 (10) | 73 (6) | 75 (6) |
| Di (2-etilhexil) fosfato + tributilfosfato (1:1, v/v) | 114 (7) | 70 (13) | 96 (8) | 97 (7) |
| **AÑADIDO A LA DISOLUCIÓN ACUOSA DONADORA** | | | | |
| **Sulfatos** | | | | |
| Ácido 1-octano sulfónico | 22 (29) | - | 8 (35) | 12 (36) |
| Ácido 1-heptano sulfónico | 6 (22) | - | 3 (21) | 5 (20) |
| **Carboxílicos** | | | | |
| Ácido octanoico | - | - | 2 (6) | 4 (24) |

### V.2.1.1.3. Valoración general de las metodologías propuestas basadas en el empleo membranas líquidas soportadas

En la **Tabla 4** se comparan los métodos de extracción propuestos haciendo referencia a aspectos tales como caraterísticas de la muestra y de la fase aceptora, los analitos determinados, si ha sido necesaria o no una etapa de dilución, las características de la membrana líquida soportada, el número de muestras que pueden procesarse de forma

simultánea, tiempo de extracción, modo de agitación y si se han realizado o no estudios complementarios de farmacocinética. Se ha realizado también una comparativa de las características analíticas de las metodologías.

**Tabla 4**. Comparación de las metodologías propuestas en los Capítulos 7 y 8.

| | **Capítulo 7** | **Capítulo 8** |
|---|---|---|
| Muestra | Saliva (2mL; pH 1-4 con HCl 1M) | Plasma (0.5 mL; dilución 1:1 con HCl 125 mM) |
| Dilución | No | Si |
| Analito | Paracetamol | Antiinflamatorios |
| Fase aceptora | NaOH 10mM (50 µL) | $NH_4OH$ 25mM (50 µL) |
| Membrana líquida soportada | PTFE de porosidad 0.5 µm y 100 µm espesor, impregnada con 5 µL 1-octanol | PP de 100 µm de espesor, impregnada con 2 µL dihexileter |
| Número de muestras procesadas simultáneamente | 3 | 96 |
| Tiempo de extracción (min) | 30 | 15 – 60 |
| Modo de agitación | Vórtex | Plataforma vibratoria |
| Estudio farmacocinético | Si | No |
| Instrumentación | HPLC-UV | HPLC-DAD |
| LD (ng/mL) | 0.5 | 6.4-22.9 |
| DER (%) | <14.2 | <22 |
| Recuperación absoluta (%) | 25 | 100 |
| Recuperación relativa (%) | 93 | 79-130 |

Por último, en la **Tabla 5** resumen las debilidades, amenazas, fortalezas y oportunidades asociadas a cada método de extracción.

**Tabla 5**. Análisis DAFO de las metodologías desarrolladas en los Capítulos 7 y 8.

| | **D**ebilidades | **A**menazas | **F**ortalezas | **O**portunidades |
|---|---|---|---|---|
| **Capítulo 7** | Bajos factores de recuperación absoluta | Difícil automatización | Adecuada para bioanálisis (<2mL)<br><br>Alta selectividad<br><br>Extractos limpios compatibles con LC | Aplicable a otro tipo de matrices como leche materna liofilizada |
| **Capítulo 8** | Bajos factores de preconcentración | Dificilmente adaptable al análisis de muestras ambientales | Adecuada para bioanálisis (<1mL)<br><br>Alta selectividad<br><br>Extractos limpios compatibles con LC<br><br>Procesa hasta 96 muestras de forma simultánea | Potencial de ser automatizada e implementada en laboratorios de rutina<br><br>Potencial de ser aplicada a la determinación de compuestos polares<br><br>Facilmente implementable en bandejas de 384 |

## V.2.2. NUEVAS FASES EXTRACTIVAS

El recubrimiento es un aspecto fundamental cuando nos referimos a la microextracción en fase sólida. El principal reto reside en el procedimiento de síntesis empleado para formar el recubrimiento, es decir, la tecnología empleada para inmovilizar de manera homogénea, reproducible y estable el sorbente (responsable de la extracción) sobre la superficie (sustrato o plataforma). Debido a que generalmente no existe una fuerte unión química

entre el polímero orgánico y el sustrato, estos sorbentes presentan una estabilidad térmica y mecánica relativamente baja, lo que limita sus aplicaciones en gran medida.

Para explotar el enorme potencial de las técnicas de microextracción, se han de diseñar fases extractivas que maximicen tanto la termodinámica como la cinética del proceso extractivo. En otras palabras, se requieren materiales novedosos con potencial en la preparación de muestras y en el desarrollo de procedimientos analíticos.

### V.2.2.1. Características de las fases extractivas sintéticas (*fabric phase*)

Las fases extractivas sintéticas (*fabric phase sorptive extraction*, FPSE) han sido desarrolladas por los profesores Kenneth G. Furton y Abuzar Kabir [22] de la Universidad de Florida (EEUU). Estos materiales combinan los principios de la SPME (extracciones basadas en el equilibrio) y de la SPE (extracciones exhaustivas) en una única plataforma tecnológica.

Las *fabric phase* están constituidas por un sustrato flexible sobre el que se sintetiza mediante tecnología sol-gel el sorbente, que es un recubrimiento híbrido orgánico-inorgánico. Este sorbente se encuentra homogéneamente distribuido y fuertemente unido a la plataforma mediante enlaces covalentes, lo que le confiere alta estabilidad [23]. Es importante destacar que, mientras en otros sistemas de microextracción el sustrato es inerte, en las *fabric phase* el sustrato contribuye a la polaridad final, haciéndolas más hidrofóbicas o hidrofílicas.

Los sustratos más empleados son el de poliéster (hidrofóbico) y el de celulosa (hidrofílico). Para sintetizar una *fabric phase* que se pueda emplear para la extracción de compuestos no polares, se utiliza el poli(dimetildifenilsiloxano) (PDMDPS), que es un polímero orgánico no polar, sobre el sustrato de poliéster. Por el contrario, el sustrato de celulosa se utiliza para albergar los polímeros orgánicos de polaridad media, como el poli(tetrahidrofurano) (PTHF), y de polaridad elevada como el polietilenglicol (PEG), dando como resultado medios de extracción de polaridad media y elevada, respectivamente (ver **Figura 7**). Se

trata por tanto de un material de polaridad modulable, es decir, mediante la correcta selección del sustrato y sorbente implicados puede adaptarse a la resolución de problemas analíticos de diferente naturaleza.

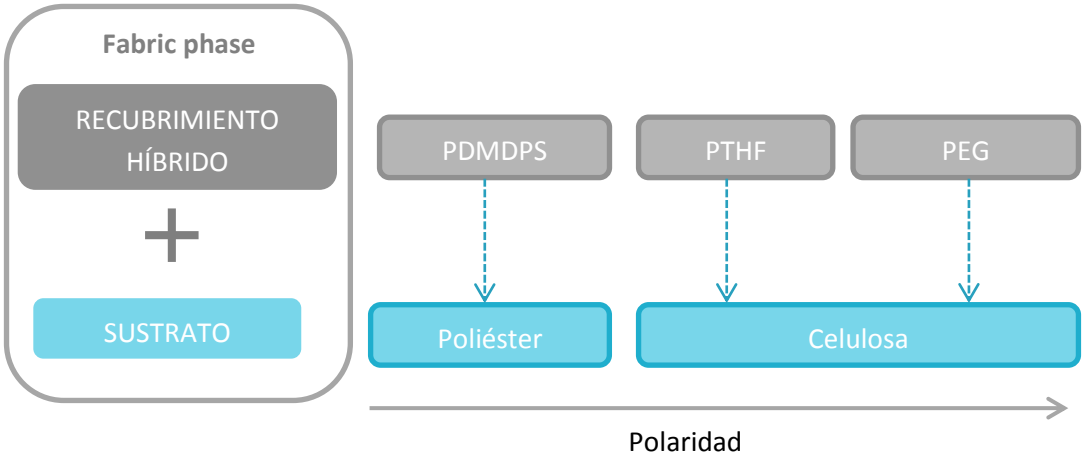


**Figura 7**. Esquema de los elementos que constituyen la *fabric phase*.

Otra característica importante de las *fabric phase* es su elevada capacidad de extracción. Su carácter poroso facilita un mayor flujo de muestra a su través, mejorando la partición de analitos entre la muestra y el sorbente. El resultado final es un material estable, versátil, flexible y con un gran potencial.

Las ventajas que ofrece la FPSE sobre otras técnicas de extracción basadas en sorción convencionales, se citan a continuación:

- La integración de SPE y SPME en FPSE hace que cualquier sorbente de los utilizados en ambas técnicas pueda utilizarse en FPSE, lo que se materializa en la posibilidad de utilizar cientos de sorbentes diferentes.

- El sorbente híbrido inorgánico-orgánico ha demostrado tener alta estabilidad mecánica y química. Además, la unión covalente entre el sustrato y el sorbente permite que se emplee cualquier tipo de disolvente para la elución de los analitos después de la extracción.
- La elevada permeabilidad del sustrato, facilita que se alcance rápidamente el equilibrio y tengan elevada capacidad de extracción.
- Se emplean sustratos que complementan la polaridad de la *fabric phase* final.
- Son aptas en la preparación de muestras complejas que contengan péptidos, biomoléculas o proteínas.

### V.2.2.2. Microextracción en *fabric phase* agitada

Con el objetivo de continuar explotando el potencial de las *fabric phase*, se firmó un acuerdo de colaboración entre nuestro grupo de investigación y el del Prof. Furton. Se optó por utilizar la configuración del sistema de extracción con membrana agitada [24], pero reemplazando la membrana de PTFE por la *fabric phase* (ver **Figura 8**) y se aplicó de forma satisfactoria a la resolución de problemas analíticos en matrices ambientales (**Capítulo 4**) y biológicas (**Capítulo 5**). De esta manera, se combinan las ventajas de los sistemas de microextracción con agitación integrada y los de nuevas fases sorbentes, *fabric phase*.

**Figura 8**. Sistema de extracción con *fabric phase* agitada.

En el primer trabajo experimental, la idea original fue estudiar la versatilidad de las tres *fabric phase* que nos suministraron: polidimetildifenilsiloxano (PDMDPS), politetrahidrofurano (PTHF) y polietilenglicol (PEG). Cada *fabric phase* se evaluó con tres familias diferentes de compuestos (triazinas, hidrocarburos policíclicos aromáticos y clorofenoles). Basándonos en nuestra experiencia previa en técnicas de microextracción, se seleccionaron unas condiciones experimentales iniciales comunes para las tres familias de compuestos, con algunas pequeñas diferencias que se comentan a continuación. Se utilizó un volumen de muestra de 100 mL, y una concentración de analito de 50 ng/mL. Para la extracción de las triazinas, se ajustó la fuerza iónica con un 5% de NaCl (w/v); para la extracción de los clorofenoles, se ajustó el pH de la muestra a 3 con HCl 1M. Antes de realizar la extracción, la *fabric phase* agitada se acondicionó con metanol y se sumergió en la muestra, agitándose a 1100 rpm durante 30 min para que tenga lugar la extracción. Una vez finalizada, se realizó la elución de los analitos de la *fabric phase* con 1 mL de metanol durante 5 min mediante agitación magnética. Finalmente, para aumentar la sensibilidad, los extractos obtenidos se evaporaron y redisolvieron en 50 µL de metanol y se analizaron mediante UPLC-DAD.

En la **Tabla 6**, se resumen las eficiencias de la extracción bajo las condiciones anteriormente descritas, expresadas como recuperaciones absolutas (AER), para las tres *fabric phase* y las tres familias de compuestos. En base a los resultados obtenidos, se seleccionó la determinación de triazinas mediante PEG como problema analítico para continuar con la evaluación de este material.

**Tabla 6**. Recuperaciones absolutas obtenidas utilizando distintas *fabric phase* con tres familias de compuestos

| Analito | Recuperación absoluta (%) Tipo de *fabric phase* | | |
|---|---|---|---|
| | **PTHF** | **PDMDPS** | **PEG** |
| **TRIAZINAS** | | | |
| Simacina | 4.4 | 0.6 | 17.8 |
| Atracina | 8.0 | 1.1 | 27.4 |
| Secbumetón | 13.1 | 1.7 | 39.6 |
| Terbumetón | 10.4 | 4.9 | 47.6 |
| Propacina | 9.7 | 6.2 | 40.3 |
| Prometrín | 18.4 | 6.3 | 53.0 |
| Terbumetrín | 22.8 | 9.4 | 58.8 |
| **PAHs** | | | |
| Naftaleno | 7.7 | 2.2 | 20.1 |
| Acenaftaleno | 11.9 | 6.8 | 32.9 |
| Fluoreno | 14.5 | 10.4 | 35.7 |
| Acenafteno | 22.1 | 20.8 | 49.5 |
| Fenantreno | 21.4 | 23.5 | 45.1 |
| Antraceno | 23.9 | 26.7 | 38.5 |
| Fluoranteno | 31.0 | 30.9 | 46.5 |
| Pireno | 24.9 | 32.5 | 41.9 |
| Criseno | 18.2 | 25.2 | 15.6 |
| Benzo (a) antraceno | 13.2 | 27.4 | 11.1 |
| Benzo (b) fluoranteno | 25.5 | 27.9 | 28.7 |
| Benzo (k) fluoranteno | 18.6 | 17.8 | 18.7 |
| Benzo (a) pireno | 19.9 | 23.5 | 22.5 |
| Dibenzo (a,h) antraceno | 10.6 | 18.4 | 8.1 |
| **CLOROFENOLES** | | | |
| 2-Clorofenol | 1.4 | 0 | 3.2 |
| 3- Clorofenol | 1.2 | 0 | 8.1 |
| 4- Clorofenol | 1.6 | 0.8 | 4.8 |
| 2,3-Diclorofenol | 4.3 | 1.1 | 14.4 |
| 2,6-Diclorofenol | 4.3 | 1.4 | 12.5 |
| 3,4-Diclorofenol | 7.2 | 2.8 | 17.9 |
| 2,4,5-Triclorofenol | 8.4 | 4.2 | 15.4 |
| 2,4,6-Triclorofenol | 11.8 | 10.5 | 22.3 |
| Pentaclorofenol | 2.8 | 0.1 | 7.4 |

El procedimiento de síntesis de las *fabric phase* con recubrimiento sol-gel PEG, se ha descrito en detalle en el **Capítulo 4**. En esencia, consta de tres etapas: (i) pretratamiento del sustrato (celulosa) con distintas disoluciones para activarlo; (ii) preparación de la disolución sol (compuesta por el polímero, precursor, medio orgánico y un catalizador sol-gel); y finalmente (iii) creación del recubrimiento sol-gel sobre el sustrato, para lo cual se introduce el sustrato acondicionado en la disolución sol. Las *fabric phase* obtenidas se caracterizaron mediante SEM, observándose que el recubrimiento sol-gel se encontraba uniformemente distribuido en las tres dimensiones del sustrato.

El estudio de las variables que afectan a la eficiencia de la extracción se realizó mediante el análisis de estándares acuosos de triazinas a una concentración de 50 µg/L, teniéndose en cuenta la fuerza iónica, el volumen de muestra, la agitación, el tiempo de extracción y la elución. Tanto la optimización como la validación se llevaron a cabo mediante UPLC-DAD, pero teniendo en cuenta que LC-MS/MS es la técnica de referencia en el ámbito del análisis ambiental, se realizó una validación complementaria utilizando esta instrumentación. La metodología propuesta con UPLC-DAD se extrapoló a LC-MS/MS, observándose que en este último caso era necesario diluir el extracto final (50 µL de metanol) en 50 µL de 5mM acetato amónico para mejorar la separación cromatográfica.

En la **Tabla 7** se resumen las condiciones experimentales y los datos más relevantes de la validación obtenidos en los **Capítulos 4** y **5**. En ambos casos se utilizó la *fabric phase* de celulosa con recubrimiento sol-gel de PEG. En el caso de las muestras de orina, es necesario un tratamiento previo antes de realizar la extracción para romper la unión de las benzofenonas con los glucurónidos, y puede hacerse de dos maneras: (i) utilizando la enzima β-glucuronidasa o (ii) mediante un tratamiento de calor y ácido. En este caso nos decantamos por la segunda alternativa ya que es la más económica. Fue necesario también realizar una dilución 1:9 para evitar el efecto matriz.

**Tabla 7.** Comparación de las metodologías propuestas en los Capítulos 4 y 5.

| | **Capítulo 4** | | **Capítulo 5** |
|---|---|---|---|
| *Fabric phase* seleccionada | PEG | | PEG |
| Muestra | Aguas ambientales (100 mL) | | Orina (5 mL). Diluída 10 veces |
| Analitos | Triazinas | | Benzofenonas |
| Tratamiento previo | No | | Si |
| Fuerza iónica | 50 g/L | | No |
| Dilución | No | | Si |
| Tiempo de extracción | 60 | | 45 |
| Velocidad de agitación | 1100 | | 1100 |
| Elución | Metanol, 1mL, 5 min | | Metanol, 1mL, 5 min |
| Instrumentación | UPLC-DAD | LC-MS/MS | LC-MS/MS |
| LD (µg/L) | 0.26-1.5 | 0.015-0.026 | 0.19-0.42 |
| RSD (%) | 11.8 | 10.8 | 11.3 |
| Recuperación absoluta (%) | 22.2-70.5 | | 11-17 |
| Recuperación relativa (%) | 75-126 | | 75.2-109.0 |

Finalmente, en la **Tabla 8** se realiza un análisis DAFO conjunto de las dos metodologías basadas en la microextracción en *fabric phase* agitada.

**Tabla 8**. Análisis DAFO de las metodologías desarrolladas utilizando la microextracción en *fabric phase* agitada.

| **D**ebilidades | **A**menazas | **F**ortalezas | **O**portunidades |
|---|---|---|---|
| Difícil aplicación a muestras de disponibilidad limitada | Dificil automatización | Sorbente distribuído homogeneamente<br><br>Síntesis reproducible<br><br>Elevada capacidad de extracción | Herramienta versátil |

## V.3. COMPARACIÓN DE LAS METODOLOGÍAS PROPUESTAS

En este último bloque, se va a realizar una comparación global de las metodologías desarrolladas empleando como indicadores de la calidad del método las propiedades analíticas básicas (precisión, sensibilidad y selectividad). También se han tenido en cuenta otros factores como el rendimiento, el coste, la versatilidad, el grado de miniaturización y automatización, el análisis *in situ* y la seguridad para el operador. Se ha establecido una escala arbitraria de 1 a 5, siendo 1 el valor más bajo y 5 el de mayor satisfacción. La comparación de los métodos se recoge en la **Tabla 9**.

Los criterios seguidos se resumen a continuación. La *precisión* se ha valorado teniendo en cuenta la desviación estándar relativa, asociada a cada metodología. La *sensibilidad* se ha está condicionada por la técnica instrumental empleada, así como por el volumen de muestra usado en cada caso. Al valorar la *selectividad* se ha optado por dar una puntuación alta a todas las metodologías ya que ésta viene determinada por la técnica instrumental utilizada. En el caso de GC/MS se han seleccionado las m/z más adecuadas, en LC-MS/MS las transiciones específicas de cuantificación e identificación para cada analito y en el caso de LC-UV/Vis la longitud de onda y el tiempo de retención. No obstante, se ha considerado que aquellas metodologías que emplean membranas como separadoras de fases merecen la máxima puntuación ya que las membranas aportan una selectividad extra, ya que actúan como barrera evitando el paso de biomoléculas, proteínas y sales al extracto final

En el *rendimiento,* se ha tenido en cuenta el número de muestras que pueden analizarse por unidad de tiempo. En cuanto al *coste,* se tuvo en cuenta el grado de reusabilidad y el consumo de reactivos, disolventes y materiales. La *versatilidad* se ha valorado de acuerdo a las perspectivas futuras previstas para cada metodología y su aplicabilidad a la resolución de otros problemas analíticos. Todas las metodologías poseen elevado *grado de miniaturización* y en cuanto al *grado de automatización* se ha valorado de forma subjetiva de acuerdo a las expectativas y a la posibilidad de su incorporación en el análisis de rutina.

En el análisis *in situ* se ha valorado la posibilidad de extrapolar las metodológias propuestas al análisis de campo. Al puntuar la *seguridad del operador* se ha tenido en cuenta el empleo de disolventes o reactivos tóxicos y el volumen utilizado de los mismos.

**Tabla 9**. Comparación de las metodologías presentadas en esta Memoria.

| | | **Técnicas de microextracción desarrolladas en la Tesis Doctoral** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **Capítulo 1** SFME | **Capítulo 2** Stir o-NHs disk | **Capítulos 4 y 5** SFPSE | **Capítulo 6** Stir octadecyl disk | **Capítulo 7** SM-LLLME | **Capítulo 8** PALME |
| Modalidad | SPME | X | X | X | | | |
| | LPME | | | | X | X | X |
| Configuración | Disco | X | X | | X | | |
| | Membrana | | | X | | X | X |
| Propiedades analíticas básicas | Sensibilidad | 5 | 4 | 4 | 4 | 3 | 1 |
| | Selectividad | 4 | 4 | 4 | 4 | 5 | 5 |
| | Precisión | 5 | 3 | 4 | 5 | 3 | 2 |
| Otros factores | Rendimiento | 2 | 4 | 2 | 2 | 4 | 5 |
| | Coste | 5 | 2 | 5 | 2 | 5 | 5 |
| | Versatilidad | 1 | 5 | 5 | 4 | 5 | 4 |
| | Grado de miniaturización | 2 | 1 | 3 | 1 | 4 | 5 |
| | Grado de automatización | 1 | 1 | 3 | 1 | 1 | 5 |
| | Análisis *in situ* | 1 | 5 | 1 | 1 | 1 | 1 |
| | Seguridad para el operador | 5 | 2 | 3 | 2 | 3 | 3 |
| **PUNTUACIÓN GLOBAL** | | 31 | 31 | 34 | 26 | 34 | 36 |

Atendiendo a la valoración realizada en la **Tabla 9**, se puede establecer una clasificación, siempre teniendo en cuenta que es una comparativa entre los métodos propuestos y que muchos aspectos están valorados de forma subjetiva. De hecho, a pesar de que un método no obtenga la máxima puntuación, podría ser más adecuado para un determinado problema analítico porque habría que tener en cuenta otros factores tales como el tipo de muestra que se va a analizar o la polaridad de los compuestos.

El método peor valorado ha sido la microextracción en fase líquida con discos derivatizados con $C_{18}$ (**Capítulo 6**). Se trata de una metodología sencilla, precisa y muy versátil. Sin embargo, se le ha otorgado baja puntuación al grado de miniaturización y a la seguridad para el operador ya que emplea un volumen relativamente grande de disolvente orgánico

(300 µL), especialmente si se compara con otras metodologías propuestas que utilizan tan solo 2-3 µL.

El siguiente puesto de la clasificación lo comparten la microextracción con frita agitada (**Capítulo 1**) y la metodología basada en discos agitados modificado con nanocuernos de carbono (**Capítulo 2**). El primero de ellos tiene en su contra la escasa versatilidad de la técnica, que restringe su uso a la determinación de compuestos no polares, resultando además muy complicado la derivatización de la frita. En cuanto a la segunda metodología, puede afirmarse que es precisa, muy versátil y además la única con posibilidad de llevar a cabo extracciones *in situ*. Sin embargo, ya que el procedimiento de síntesis requiere el uso de diferentes reactivos, se ha considerado el coste y la seguridad para el operador como uno de sus puntos más débiles.

A continuación se encuentran la microextracción en *fabric phase* agitada (**Capítulos 4** y **5**), y la microextracción en fase líquida con membrana agitada (**Capítulo 7**). De la primera de ellas cabe destacar su bajo coste de síntesis y alta versatilidad ya que en función de los sustratos y recubrimientos empleados, pueden obtenerse medios de extracción de distinta naturaleza. Se le ha otorgado una puntuación intermedia en el grado de automatización ya se espera que en un futuro pueda ser acoplada a equipos comerciales, de una forma similar a la microextracción en capa fina (thin film microextraction, TFME). En cuanto a la segunda, su versatilidad queda patente en las distintas modificaciones de la técnica que hacen posible su acoplamiento con técnicas espectroscópicas y cromatográficas, que puedan analizarse muestras sólidas y líquidas (tanto en la modalidad de dos como tres fases). La sensibilidad, la precisión y la falta de automatización son los puntos débiles de esta metodología.

El primer puesto de la clasificación es para el sistema de microextracción con membranas agitadas en paralelo (**Capítulo 8**). Es la única capaz de procesar hasta 96 muestras de forma simultánea, por ello se le ha otorgado la máxima puntuación en el rendimiento. También tiene a su favor que actualmente se está trabajando en su completa

automatización. Es versátil puesto que en ensayos preliminares se ha demostrado su potencial para determinar compuestos polares. En su contra, la precisión de todos los analitos no es la ideal y que dado el bajo volumen de muestra que emplea, la sensibilidad del método es baja. Este hecho podría solventar se si se utilizara en el análisis LC-MS/MS.

Finalmente, en la **Figura 9** se engloban todas las metodologías propuestas y se resumen las características más importantes de cada una.

**Figura 9**. Unidades de extracción con agitación integrada desarrolladas en la presente Memoria.

Con esto se cierra este apartado de Resultados y Discusión donde se han abordado de manera comparativa el trabajo experimental desarrollado en esta Memoria de Tesis Doctoral.

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BLOQUE VI

# CONCLUSIONES

*CONCLUSIONS*

El tratamiento de muestra es el cuello de botella de la mayoría de procedimientos analíticos. La Química Analítica actual centra gran parte de sus esfuerzos en el diseño y optimización de metodologías más simples, automatizadas, baratas, rápidas y seguras. Durante el desarrollo de esta Tesis Doctoral, se ha profundizado en el diseño de metodologías analíticas innovadoras para el desarrollo y mejora de las técnicas de tratamiento previo de la muestra.

Esta Memoria consta de dos grandes bloques temáticos, ambos orientados al desarrollo de unidades de extracción con agitación integrada. La investigación también ha estado encaminada a la síntesis y evaluación de nuevos materiales sorbentes aplicados a técnicas de microextracción Así, el Bloque III de la Memoria se centra en el desarrollo de nuevas metodologías analíticas basadas en los principios de la microextracción en fase sólida. El Bloque IV recoge los dispositivos desarrollados en el contexto de las técnicas de microextracción en fase líquida. En esta sección se van a exponer las principales conclusiones derivadas de la investigación desarrollada a lo largo de estos cuatro años, en función del bloque temático al que pertenece, desde un punto de vista operacional y analítico.

En el contexto de la **microextracción en fase sólida**, se han desarrollado tres estrategias para la determinación de compuestos orgánicos:

- ✓ Se ha desarrollado una nueva modalidad de microextracción denominada microextracción en frita agitada, basada en el uso de fritas comerciales de polietileno como medio sorbente. Para ello, las fritas se perforaron con un alambre que permite su agitación magnética durante el proceso de extracción, lo que facilita la transferencia de los analitos desde la muestra hasta el medio de extracción. Se trata de un procedimiento analítico muy simple, de bajo coste y respetuoso con el medio ambiente que se ha propuesto para la determinación de compuestos orgánicos volátiles en muestras de agua, sin necesidad de filtración previa. Los

analitos retenidos en la frita se desorbieron térmicamente y se analizaron mediante GC/MS. Esta metodología sería fácilmente trasladable a cromatografía de líquidos si se realiza una elución química.

- ✓ Se ha desarrollado una nueva herramienta basada en la inmovilización de nanocuernos de carbono oxidados sobre los poros de un disco de borosilicato. Los discos resultantes se caracterizaron mediante SEM, y se aplicó a la determinación de benzofenona-3 en muestras de agua piscina. El disco sintetizado se acopló a un taladro portátil para hacer posible su agitación. La elevada velocidad de agitación que proporciona el taladro unido a la excelente capacidad sorbente de las nanopartículas empleadas, hizo posible homogeneizar un volumen elevado de muestra. Indudablemente, esta técnica proporciona nuevas oportunidades para el análisis *in situ* de compuestos orgánicos.

- ✓ Se ha desarrollado una nueva modalidad de extracción llamada microextracción en *fabric phase* agitada. Esta técnica está basada en el uso de *fabric phase* como material sorbente, el cual está fijado en un dispositivo que permite la agitación magnética del mismo. Las *fabric phase* empleadas están constituidas por un soporte flexible de celulosa sobre el cual se ha sintetizado un recubrimiento híbrido orgánico-inorgánico de PEG, y se ha caracterizado mediante SEM. Entre las múltiples ventajas de este novedoso material sorbente, cabe destacar que tanto el soporte como el recubrimiento contribuyen a la polaridad final de la *fabric phase*. Se han desarrollado dos metodologías analíticas aplicadas a la determinación de triazinas en muestras de agua y benzofenonas en muestras de orina mediante LC.

En lo que respecta a la **microextracción en fase líquida**, cabe destacar que:

- ✓ Se ha desarrollado una nueva metodología basada en discos de borosilicato modificados con grupos octadecilo para la estabilización del líquido extractante. El

disco resultante se caracterizó mediante un rápido ensayo colorimétrico con fenolftaleína que confirmó que la derivatización se había llevado a cabo. Se realizó también un espectro de IR, que dio lugar a bandas de baja intensidad correspondientes a las vibraciones C-H de la fase octadecil. Es precisamente esta baja intensidad la que explicaría el aspecto fundamental de esta aplicación, y es que el recubrimiento con $C_{18}$ es suficiente para estabilizar al disolvente orgánico no polar pero no tan elevado como para ser él mismo el responsable de la extracción. Por lo tanto, para utilizar el $C_{18}$ bajo los principios de la µ-SPE debería de incrementarse el recubrimiento de la fase activa. El disco resultante, se inserta en una unidad especialmente diseñada que consta de un imán de gran potencia que permite la agitación magnética del mismo. Esta técnica es ideal para procesar grandes volúmenes de muestra, y por ende, los factores de preconcentración que se obtienen son muy elevados. Además, dependiendo del grupo funcional inmovilizado, se podrían estabilizar disolventes orgánicos de diferente naturaleza, adecuando la unidad de microextracción al problema analítico en cuestión.

- ✓ Se ha ampliado el campo de aplicación de la microextracción con membrana agitada al proponer un nuevo diseño de la unidad de extracción original. Con la modificación llevada a cabo, la muestra queda integrada dentro del sistema, haciendo posible que puedan emplearse volúmenes pequeños de muestra. Este hecho es de especial interés ya que posibilita que esta metodología se pueda emplear con muestras valiosas o muestras de las que solo se dispone de unos pocos mililitros. También resulta ser una técnica bastante selectiva, y se ha podido llevar a cabo determinaciones en muestras complejas, como es el caso de los biofluidos. Esta selectividad viene determinada por la porosidad de la membrana, que impide el paso de biomoléculas, así como por el gradiente de pH establecido a ambos lados de la membrana que restringe el paso a compuestos no polares ionizables. Los extractos obtenidos son directamente compatibles con LC.

- ✓ Se ha ampliado la versatilidad de la microextracción con membranas agitadas en paralelo, empleándose por primera vez con fármacos de naturaleza ácida. De todas las metodologías desarrolladas en la Memoria, ésta es la única que cuenta con una tecnología capaz de procesar hasta 96 muestras de forma simultánea. Si bien es cierto que para la realización de este trabajo experimental se ha utilizado un prototipo diseñado en el laboratorio, actualmente se está desarrollando una plataforma comercial con vistas a automatizar la metodología para que pueda ser usada en los laboratorios de rutina. Además, se ha comprobado que las membranas de PP eran las mejores candidatas ya que las de PVDF daban lugar a interacciones no específicas con los analitos. Se trata de una metodología sencilla, versátil y respetuosa con el medio ambiente ya que solo requiere de 2 µL de disolvente orgánico por muestra. Resulta ser también una opción bastante económica en el tratamiento de muestras biológicas ya que el coste por muestra es de aproximadamente 0.25 euros. Al igual que la microextracción con membrana agitada, ésta es también una técnica muy selectiva, ya que los extractos de las muestras de plasma obtenidos están libres de interferencias y son compatibles con LC. A pesar de que esta técnica no ofrece factores de preconcentración elevados, permite cuantificar de manera satisfactoria en el intervalo terapeútico los compuestos estudiados en muestras de plasma.

Finalmente, cabe destacar que todas las alternativas desarrolladas durante la realización de esta Tesis Doctoral se han evaluado mediante la aplicación a muestras de agua de diferente naturaleza (río, arroyo, pozo, grifo, piscinas y embotellada) o biológicas (saliva, plasma y orina). En todos los casos se han alcanzado buenos valores en cuanto a los límites de detección y precisión. Asímismo, los resultados para muestras fortificadas de agua cumplieron los requisitos establecidos por la US-EPA (recuperación comprendida entre el 70-130%). En cuanto a las muestras biológicas, la validación se realizó de acuerdo a la guía FDA.

*Sample treatment is the bottleneck for most analytical procedures. Current Analytical Chemistry is mainly focused on the design and optimization of simple, automated, cheap, fast and environmentally-friendly analytical methodologies. The research carried out in this Doctoral Thesis was directly towards the design of innovative analytical methodologies for the development of new and improved sample treatment.*

*This Report consists of two main thematic blocks, both aimed at developing novel extraction devices with integrated stirring. The first block (Section III) is based on the development of new analytical methodologies under the solid phase microextraction principles. The second block (Section IV) includes the development of extraction devices in the liquid phase microextraction field.*

*In this section, the main conclusions that can be drawn from the research developed during the Doctoral Thesis will be presented according to the block they belong to, from an operational and analytical point of view.*

*In the solid phase microextraction context, three different strategies for the determination of organic compounds have been developed:*

- ✓ *A new methodology called stir frit microextraction, which is based on the use of commercially available polyethylene frits as active sorbent medium has been developed. For this purpose, frits are pierced with an iron wire to allow its magnetic stirring during the extraction process, which facilitates the transfer of analytes from the sample to the extraction medium. The analytical procedure is very simple, low-cost and environmentally-friendly. It has been proposed for determination of volatile organic compounds in unfiltered environmental water samples. The analytes retained on the frit were thermally desorbed and analyzed by GC/MS. This methodology would be easily compatible with liquid chromatography if a chemical elution is performed.*

- ✓ *A new tool based on the immobilization of oxidized carbon nanohorns on the pores of a borosilicate disk has been developed. The final disks were characterized by SEM, and applied to the determination of benzophenone-3 in swimming pool water samples. The synthesized disk was attached to a portable drill that enables its agitation in the sample. The high stirring speed provided by the drill together with the excellent sorbent properties of the nanoparticles employed, made possible to homogenize a large sample volume. Undoubtedly, this proposal opens door for on-site extraction of organic compounds.*

- ✓ *A new methodology called stir fabric phase microextraction has been developed. The approach is based on the use of fabric phase as sorbent material, which is fixed in a stirring device. The fabric phase employed is made of a flexible support (cellulose) used as the host matrix for a hybrid organic-inorganic coating of PEG. The final fabric phase was characterized by SEM. Among the advantages of this novel sorbent material, the join contribution of both, the support and the coating to the final polarity of the fabric phase can be highlighted. This extraction unit has been applied to the determination of triazines in water samples and benzophenones in urine samples using LC.*

*Concerning the liquid phase microextraction, it should be highlight that:*

- ✓ *A new methodology based on borosilicate disks modified with octadecyl groups which act as support of the organic extracting solvent thanks to hydrophobic interactions has been developed. The resulting disk was characterized by means of a fast colorimetric assay with phenolphthalein which confirmed that the derivatization was carried out. The IR spectrum showed low intensity bands corresponding to the presence of the octadecyl phase. It is precisely this low intensity which would explain the fundamental aspect of this application. The coating is enough to stabilize the nonpolar organic solvent but not so high as to be*

*itself the responsible for the extraction. Therefore, this methodology would be easily applicable under the µSPE principles by increasing the active phase. The resultant disk is inserted into a tailored unit consisting of a plastic body with a strong magnet that allows its magnetic stirring. This technique is ideal for processing large sample volumes and therefore high preconcentration factors may be achieved. In addition to this, depending on the functional group immobilized, solvent of different nature may be stabilized. This fact permits the microextraction unit to be adapted to any analytical problem.*

- ✓ *The scope of the stir membrane microextraction has been expanded since a new design of the original extraction device has been described. The proposal integrates the sample within the extraction device reducing the sample volume to the low mL range. This fact is of a great importance in the bioanalytical field as it allows analyzing small sample volume. Moreover, this is a very selective procedure, and it has been possible to carry out measurements in complex samples, such as biofluids. The artificial liquid membrane employed serves as a very efficient and high selectivity and sample clean-up can be obtained. The extracts are directly compatible with LC.*

- ✓ *The versatility of parallel artificial liquid membrane extraction with acidic drugs has been demonstrated. Among all the methodologies developed in the Report, this is the only one with a technology capable of processing up to 96 samples simultaneously. It is true that this experimental study was performed using a prototype designed on the laboratory. Nowadays, efforts are focused on developing a commercial platform in order to automate the methodology with the goal to be used in routine analysis. Furthermore, it was found that PP membranes were the best candidates since nonspecific interactions with the analytes were observed with PVDF membranes. It is a simple, versatile and green approach to the environment since it only requires 2 µL of organic solvent per sample. It will also be*

*a very economical choice in the treatment of biological samples because the cost per sample is about 0.25 euro. As it is the case with the latter approach, this is also a very selective technique as the extracts obtained from plasma samples are free from interferences and directly compatible with LC. Although this technique does not offer high preconcentration factors, it quantifies satisfactorily the studies compounds within the therapeutic range.*

*Finally, it should be highlighted that all the alternatives developed during this Doctoral Thesis were evaluated by applying the proposed methodologies either to waters of different nature (river, stream, well, tap, swimming pools and bottled water) or biological (saliva, plasma and urine) samples. In all cases, the limit of detection and precision achieved were adequate to the analytical problem. Moreover, the results for spiked water samples fulfill the requirements established by the US-EPA (recovery between 70-130%). As for the biological samples, validation was performed according to the FDA guideline.*

BLOQUE VII

# AUTOEVALUACIÓN CIENTÍFICA

## *SCIENTIFIC SELF-ASSESSMENT*

La perspectiva que aporta el tiempo y la experiencia adquirida, permite analizar de forma crítica el trabajo desarrollado. Además de las aportaciones más relevantes que se han expuesto en la sección anterior, también conviene mencionar aquellas limitaciones o dificultades encontradas en el desarrollo de la fase experimental, para así poder definir las futuras líneas de investigación.

$\mathcal{X}$ En cuanto a la modalidad de microextracción en frita agitada, las principales limitaciones las encontramos tanto en el material sorbente como en la etapa de desorción térmica. Por un lado, la naturaleza hidrofóbica de la frita reduce su campo de aplicación a la extracción de compuestos apolares. En cuanto a la desorción, deberían diseñarse interfaces específicas para introducir en el sistema cromatográfico la totalidad de los analitos que están retenidos en la frita, y evitar así, su dilución en el espacio de cabeza del vial. Además, la temperatura de desorción está limitada ya que la frita es un material termolábil, por lo que no podrían desorberse compuestos a temperaturas superiores a los 90 ºC sin que la frita se deteriorase. Otro aspecto a tener en cuenta en futuras aplicaciones sería recubrir/proteger la barrita magnética con la que se perfora la frita para así evitar la aparición de óxido. No se ha estudiado si este óxido podría dar lugar a interferencias no deseadas.

$\mathcal{X}$ Con respecto a la inmovilización de nanocuernos oxidados sobre los poros del disco de borosilicato, se obtuvo una buena reproducibilidad entre discos, sin embargo, se trata de una síntesis tediosa y que necesita mucho tiempo. Además, es difícil saber con exactitud la cantidad de sorbente que ha quedado inmovilizado. El método analítico desarrollado se aplicó a la determinación de un único analito, por lo tanto, sería conveniente que se llevasen a cabo estudios para la determinación simultánea de varios compuestos presentes en una misma muestra. Por otro lado, se plantea la posibilidad de utilizar esta herramienta para llevar a cabo extracciones *in situ*, sin embargo, debido a que no se encontraron muestras positivas no llegó a realizarse una comparación crítica entre

extracciones *in situ* y en el laboratorio. Finalmente, para expandir la aplicabilidad, convendría evaluar el potencial de estos discos derivatizados con otro tipo de nanopartículas.

- En la modalidad de extracción en disco de borosilicato que tiene lugar bajo los principios de la microextracción en fase líquida, sería interesante estudiar otras derivatizaciones así como el comportamiento de otros extractantes, como podrían ser los líquidos iónicos.

- Dado el gran potencial que presentan las *fabric phase*, convendría trabajar en la automatización de la técnica. Del mismo modo que la microextracción en capa fina está consolidada y existen plataformas totalmente automatizadas para la extracción simultánea de 96 y 384 muestras, el acoplamiento de *fabric phase* con este tipo de sistemas sería posible, pero está aún por desarrollar. La aplicación de este novedoso material a la resolución de problemas analíticos se encuentra en su primera etapa, así que sería importante extender su aplicabilidad a otras áreas.

- Con respecto a la microextracción con membrana agitada, no se ha estudiado la influencia que podrían tener las partículas en suspensión en el proceso de extracción. En principio podría ser negativo si se rompiera la membrana debido a posibles impactos durante la agitación. Este estudio se intentará abordar en futuras aplicaciones de esta metodología. Otro parámetro a evaluar en profundidad sería el disolvente que impregne la membrana, ya que como se ha comprobado, la naturaleza de la membrana líquida soportada es clave en la eficiencia de la extracción.

- Para poder explotar todo el potencial de la técnica de microextracción con membrana agitada en paralelo, se debería abordar su aplicación a matrices más complejas como por ejemplo la sangre.

$\mathcal{X}$ El punto débil de la mayoría de las aplicaciones desarrolladas reside en la falta de automatización lo que supone una limitación para su transferencia al análisis de rutina.

*The insights derived from the passage of time and the experience acquired, allows the critical assessment of the doctoral work carried out. In addition to the most relevant contributions previously stated, this Report concluded with the shortcoming that might be addressed in future research.*

*✗ As regards the stir frit microextraction approach, the two main limitations are related to the sorbent material and the desorption step. On the one hand, the hydrophobic nature of the frit limits its application to the extraction of nonpolar compounds. On the other hand, concerning the thermal desorption, efforts should be directed towards the design of specific interfaces to introduce the totality of the analytes retained in the frit into the chromatographic system, thus avoiding the dilution in the headspace of the vial. Furthermore, the desorption temperature is a restricted issue because of the thermo labil nature of the frit, which makes unfeasible desorbed compounds at temperatures above 90ºC without damaging the sorbent material. Another aspect to consider in future applications would be to cover or protect the iron wire so as to avoid the appearance of rust. It has not been studied whether this oxide could lead to undesirable interferences.*

*✗ Concerning the borosilicate disk, it may be interesting to study other derivatizations as well as the behavior of other extractants such as ionic liquids.*

*✗ Taking into account the great potential presented by phase fabric sorptive extraction media, effort should be directed towards automation. By way of an example, thin film microextraction is a consolidated technique with fully automated platform for simultaneous extraction of 96 and 384 samples. The implementation of fabric phase into automated systems would be also possible and therefore it should be studied. Furthermore, it would be important to extend the applicability of this novel material to other research areas.*

*✗ As regards the stir membrane microextraction approach, the influence of suspended particles in the extraction process was not evaluated. Theoretically, it could be*

*negative if the membrane is broken because of possible impacts during agitation. This study will be included in future applications of this methodology. Another key parameter to be further evaluated is the solvent employed to form the supported liquid membrane, because as it has been demonstrated, it plays a pivotal role in the extraction efficiency.*

*✗ To fully exploit the potential of parallel artificial liquid membrane extraction, it should be necessary to extend its application to more complex matrices such as whole blood.*

*✗ The weak point of most of the developed applications is the lack of automation, which is a limitation for routine analysis.*

# ANEXOS

# Producción científica

**Anexo A**

Publicaciones científicas derivadas de la Tesis Doctoral

**Anexo B**

Actividades de divulgación científica

**Anexo C**

Presentación de comunicaciones a congresos

ANEXO A

# Publicaciones científicas derivadas de la Tesis Doctoral

## ARTÍCULOS CIENTÍFICOS PUBLICADOS

## *Scientific published articles*

**1. Stir frit microextraction: An approach for the determination of volatile compounds in water by headspace-gas chromatography/mass spectrometry.**

M. Roldán-Pijuán, M.C. Alcudia-León, R. Lucena, S. Cárdenas, M. Valcárcel, *Journal of Chromatography A* 1251 (2012) 10-15.

**2. Stir membrane liquid microextraction for the determination of paracetamol in human saliva samples.**

M. Roldán-Pijuán, M. C. Alcudia-León, R. Lucena, S. Cárdenas and M. Valcárcel, *Bioanalysis* 5 (2013) 307-315.

**3. Stir octadecyl-modified borosilicate disk for the liquid phase microextraction of triazine herbicides from environmental waters.**

M. Roldán-Pijuán, R. Lucena, M.C. Alcudia-León, S. Cárdenas and M. Valcárcel. *Journal of Chromatography A* 1307 (2013) 58-65.

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M. Roldán-Pijuán, R. Lucena, S. Cárdenas, M. Valcárcel, A. Kabir, K. G. Furton. *In process.*

## CO-AUTORA DE OTROS ARTÍCULOS CIENTÍFICOS PUBLICADOS

### *Other scientific published articles co-authored*

**1. Stir-membrane solid-liquid-liquid microextraction for the determination of parabens in human breast milk samples by ultra high performance liquid chromatography-tandem mass spectrometry.**

R. Rodríguez-Gómez, M. Roldán-Pijuán, R. Lucena, S. Cárdenas, O. Ballesteros, A. Navalón and M. Valcárcel. *Journal of Chromatography A* 1354 (2014) 26-33.

Journal of Chromatography A, 1354 (2014) 26–33

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# Stir-membrane solid–liquid–liquid microextraction for the determination of parabens in human breast milk samples by ultra high performance liquid chromatography-tandem mass spectrometry

Rocío Rodríguez-Gómez [a], Mercedes Roldán-Pijuán [b], Rafael Lucena [b], Soledad Cárdenas [b], Alberto Zafra-Gómez [a], Oscar Ballesteros [a], Alberto Navalón [a], Miguel Valcárcel [b,*]

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ABSTRACT

In this article, stir-membrane solid–liquid–liquid microextraction (SM-SLLME) is tailored for the analysis of solid matrices and it has been evaluated for the determination of parabens in l breast milk samples. A three-phase microextraction mode was used for the extraction of the target compounds taking advantage of their acid–base properties. The unit allows the simultaneous extraction of the target compounds from the solid sample to an organic media and the subsequent transference of the analytes to an aqueous acceptor phase. The method includes the identification and quantification of the analytes by ultra high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). All the variables involved in the extraction procedure have been accurately studied and optimized. The analytes were detected and quantified using a triple quadrupole mass spectrometer (QqQ). The selection of two specific fragmentation transitions for each compound allowed simultaneous quantification and identification. The method has been analytically characterized on the basis of its linearity, sensitivity and precision. Limits of detection ranged from 0.1 to 0.2 ng $mL^{-1}$ with precision better than 8%, (expressed as relative standard deviation). Relative recoveries were in the range from 91 to 106% which demonstrated the applicability of the stir-membrane solid–liquid–liquid microextraction for the proposed analytical problem. Moreover, the method has been satisfactorily applied for the determination of parabens in lyophilized breast milk samples from 10 randomly selected individuals.

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## 1. Introduction

The alkyl esters of *p*-hydroxybenzoic acid (parabens, PBs) are a group of compounds widely used as bactericide and antimicrobial preservatives, especially against mold and yeast in cosmetic products, pharmaceuticals, and in food and beverage processing [1]. The biological activity of PBs is based on their inhibitory effects on membrane transport and mitochondrial function processes. These compounds are present, individually or in combination, in a large amount of commercial formulations. Although PBs have been considered for years to be relatively safe compounds with a low bioaccumulation potential [2], some studies suggest that they present a moderate endocrine disrupting activity and therefore they can cause adverse effects on humans and wildlife. In fact, the ability of PBs to disrupt physiologically important functions in both *in vitro* systems [3] and *in vivo* models [4–6] has been demonstrated. As well, the presence of non-metabolized PBs in breast cancer tissues [7] has focused the attention in their potential carcinogenic and toxic nature [2,6,8].

The human exposure to PBs may occur through ingestion, inhalation or dermal absorption. This exposure, estimated in 76 mg per day, involves different sources such as cosmetics and personal care products (50 mg/day), drugs (25 mg/day) or food (1 mg/day) [1]. After intake, PBs are metabolized by hydrolysis of the ester bond and by glucuronidation [9]. However, the parent compounds (free forms) can still be detected in biological samples such as urine [10], serum and seminal plasma [11] and human milk [12].

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## CAPÍTULOS DE LIBRO
## *Book chapters*

**1**. **Microextraction techniques based on the combination of agitation and extraction in the same device.**

M. Roldán-Pijuán, R. Lucena and S. Cárdenas. *Encyclopedia of Analytical Chemistry. Editorial John Wiley & Sons* (2015). Pruebas de imprenta corregidas enero 2015.

**2. Solid phase microextraction under the thin film format.**

M. Roldán-Pijuán, R. Lucena and S. Cárdenas and M. Valcárcel.

Editorial Bentham. E-book Analytical Microextraction Techniques. Aceptado. Prevista su publicación en el primer semestre 2015.

## ARTÍCULOS DE DIVULGACIÓN CIENTÍFICA

## *Science comunications*

**1. Microextracción líquida con membrana agitada**

M. C. Alcudia-León, M. Roldán-Pijuán, R. Lucena, S. Cárdenas, M. Valcárcel.

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ANEXO B

# Actividades de divulgación científica

GRUPO REGIONAL ANDALUZ SOCIEDAD

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# Artículo de divulgación científica

## Microextracción líquida con membrana agitada

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# MICROEXTRACCIÓN LÍQUIDA CON MEMBRANA AGITADA

M.C. Alcudia-León, M. Roldán-Pijuán, R. Lucena, S. Cárdenas, M. Valcárcel

## 1. INTRODUCCIÓN

La determinación de compuestos a baja concentración implica su aislamiento de la muestra y preconcentración antes de su análisis. Tradicionalmente, la separación y preconcentración se ha logrado mediante el empleo de técnicas tales como la extracción líquido-líquido (LLE) y la extracción en fase sólida. Generalmente, estos métodos consumen grandes cantidades de muestra y disolventes, y requieren mucho tiempo y dedicación por parte del analista. En este contexto, las investigaciones en los últimos años se han centrado en el desarrollo de estrategias que permitan reducir el tiempo de tratamiento de muestra y mejorar la eficiencia de los procesos de extracción. Las estrategias con mayor éxito se han basado en el uso de energías auxiliares como los ultrasonidos, las microondas, la radiación láser y ultravioleta, el campo eléctrico, la agitación y la temperatura entre otras.

En las últimas décadas, las membranas se han empleado en diferentes procesos de separación, como por ejemplo, la desalinización, la diálisis, la ultrafiltración, la separación de gases, la deshumidificación, la ósmosis inversa y la electrodiálisis. Por tanto, es concebible que puedan utilizarse para la separación y preconcentración en aplicaciones analíticas. Las membranas pueden ser selectivas a una determinada especie, pero principalmente funcionan como un separador de fases, controlando la transferencia de

masa entre ellas. Esto permite la preconcentración de las especies de interés y la eliminación de la matriz de la muestra.

El uso de membranas en el pretratamiento de muestra, en muchos casos, se ha convertido en una opción prioritaria. En gran parte, se debe al hecho de que facilitan la extracción sin la mezcla de fases, permitiendo el contacto continuo entre la muestra y el extractante, y evitando problemas como la formación de emulsiones. Además, existe una gran variedad de membranas comerciales que pueden clasificarse en función de su geometría, morfología, estructura, capacidad o composición.

La extracción con membrana agitada (SME) fue desarrollada en 2009 por nuestro grupo de investigación [1]. Se diseñó una novedosa unidad de extracción que incorporaba tanto la agitación magnética como una membrana polimérica en el mismo dispositivo. Esta agitación incrementa la velocidad de extracción, permitiendo alcanzar eficiencias mayores a tiempos menores. La gran variedad de membranas poliméricas disponibles en el mercado hacen de la microextracción con membrana agitada una técnica muy versátil. Además, se puede acoplar tanto a técnicas cromatográficas como espectroscópicas.

Recientemente, se ha avanzado en el estudio de la mejora de esta modalidad de extracción con vistas a incrementar la versatilidad de la misma. En este sentido, se ha desarrollado una nueva técnica de microextracción en fase líquida (LPME), la llamada microextracción líquida con membrana agitada (SM-LLME), que incluye las ventajas de la LPME y la agitación en la misma unidad, permitiendo la extracción de los analitos de un modo simple y eficiente. La SM-LLME puede operar en dos formatos diferentes, dos o tres fases, según el número de fases implicadas. La elección del formato se realiza teniendo en cuenta las características químicas de los analitos a determinar.

## 2. MICROEXTRACCIÓN LÍOUIDA CON MEMBRANA AGITADA

El efecto beneficioso de integrar la agitación y la extracción en la misma unidad se evaluó estableciendo el perfil de variación de la señal analítica con el tiempo de extracción. Este parámetro se estudió empleando la misma unidad de extracción bajo dos condiciones diferentes. En un caso, la agitación y la extracción están integradas en la misma unidad, y en el otro la agitación magnética tiene lugar externamente mientras la unidad de extracción permanece estática. Como puede observarse en la **Figura 1**, la integración permite obtener mejores resultados, ya que la agitación facilita la transferencia de los analitos desde la muestra hacia la fase aceptora.

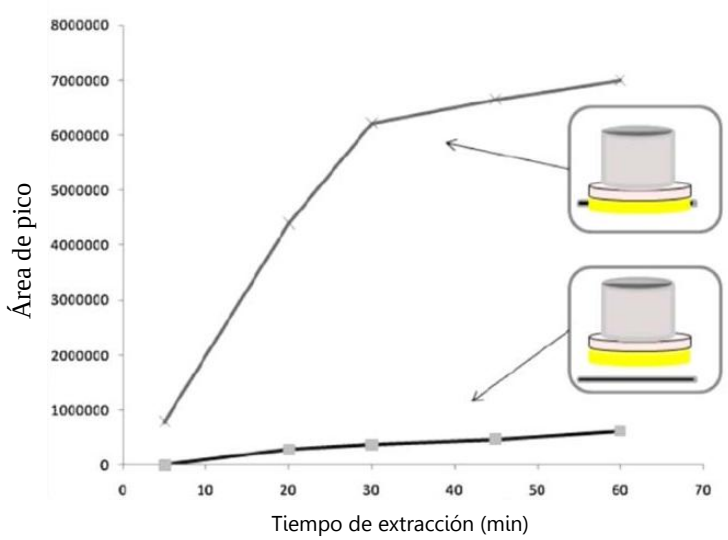


**Figura 1**. Estudio del efecto de la integración de la agitación y la extracción en la misma unidad. Más detalles en el texto. Reproducido de la referencia [2] con el permiso de Elsevier.

## 2.1. Unidad de extracción

La pieza clave de esta metodología es la unidad de extracción (**Figura 2**), que está constituida por 5 elementos básicos: (i) una barrita de hierro, (ii) un tapón comercial de politetrafluoroetileno (PTFE) para los cartuchos de SPE de 3 mL, (iii) la sección superior de uno de estos cartuchos, (iv) una membrana de PTFE, y (v) una sección de una punta de pipeta de 5 mL. El empleo de elementos comerciales en su diseño, garantiza la reproducibilidad en su construcción, aunque, por otro lado, la optimización de estos sistemas de extracción está limitada únicamente al volumen de la fase aceptora. El proceso de ensamblaje es bastante sencillo, y puede realizarse en un par de minutos. En primer lugar, el elemento interno (iii) se sella a presión con el tapón (ii), previamente perforado por la barrita de hierro (i) que es la que permite la agitación magnética de la unidad de extracción. El ensamblaje de los elementos (ii) y (iii) es crucial, ya que definen la cámara interna donde más tarde se situará el extractante. Una vez añadido éste, se deposita la membrana (iv) sobre la unidad, fijándola por desplazamiento del elemento externo (v), siendo el volumen interno de la cámara de 50 µL.

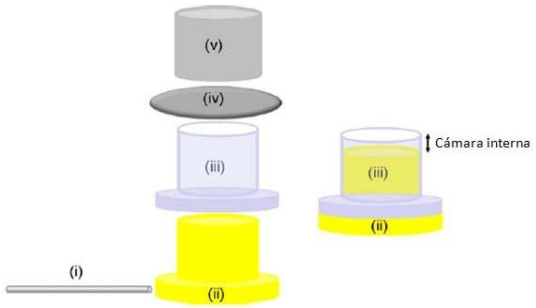


**Figura 2**. Elementos básicos de la unidad de microextracción con membrana agitada. Más detalles en el texto. Reproducido de la referencia [2] con el permiso de Elsevier.

### 2.3. Formatos

La SM-LLME puede operar en dos formatos, dos o tres fases en función del número de fases implicadas en el proceso, de tal manera que los analitos migran desde la muestra a la fase aceptora confinada en el interior de la unidad, a través de la membrana líquida soportada. Esta fase aceptora puede ser un disolvente orgánico (el mismo que se ha usado para constituir la membrana líquida soportada), dando lugar a la modalidad de dos fases; o bien una disolución acuosa ácida o alcalina, dando lugar a un sistema de extracción en tres fases.

#### 2.3.1. Microextracción líquida-líquida con membrana agitada (dos fases)

Esta nueva técnica de microextracción se caracterizó para la resolución de un problema analítico modelo: la determinación de cinco clorofenoles en agua [2]. Empleando el formato en dos fases, se llevó a cabo la extracción de los analitos con disolvente orgánico, y la derivatización in-situ de los mismos. La reacción de derivatización no sólo mejora la determinación de los clorofenoles por cromatografía de gases, sino que también actúa como fuerza impulsora en el proceso de extracción al desplazar el equilibrio. El incremento de la proporción de derivatizante en el disolvente orgánico que actúa como extractante, produce una mejora evidente en los resultados.

El disolvente orgánico empleado como extractante juega un papel importante en el procedimiento de extracción líquido-líquido; debiendo cumplir una serie de requisitos. En primer lugar, este debe presentar una elevada afinidad por los analitos de interés, para poder extraerlos de la matriz de la muestra. Además, debe ser químicamente compatible con la membrana empleada y debe presentar una baja solubilidad en agua para evitar su pérdida durante la extracción. Finalmente, también debe tenerse en cuenta su compatibilidad con la técnica instrumental empleada para el análisis.

El resto de las variables implicadas en el proceso de extracción también se identificaron y optimizaron (velocidad y tiempo de agitación, y el volumen de muestra y extractante). Trabajando en las condiciones óptimas, la metodología desarrollada permite la determinación de estos clorofenoles con límites de detección en el intervalo de 14.8 ng/L (para el 2,4,5-triclorofenol) a 22.9 ng/L (para el 3-clorofenol) con una desviación estándar relativa menor del 8.7 % (para el 2,6- diclorofenol) y factores de enriquecimiento superiores a 166.

### 2.3.2. Microextracción líquida-líquida-líquida con membrana agitada (tres fases)

El formato de tres fases se realiza en el mismo dispositivo del formato en dos fases, pero en este caso, el disolvente orgánico sólo impregna los poros de la membrana, mientras que en la cámara interna del dispositivo se localiza la fase acuosa aceptora [3]. Esta modalidad permite la extracción de los analitos que presentan algún grupo ionizable al establecer un gradiente de pH a ambos lados de la membrana líquida soportada, siendo este gradiente el que fuerza la extracción de los analitos. En función de la estructura química de los compuestos a determinar, las muestras deben ajustarse a un pH tal que los analitos se encuentren en su forma no iónica y puedan así ser extraídos por el disolvente orgánico, mientras que la fase aceptora debe presentar un pH en el que los analitos se encuentren en su forma iónica y no puedan ser reextraídos por el disolvente orgánico. Es, por tanto, este gradiente el que fuerza la extracción de los analitos desde la fase donadora a la aceptora.

Este formato se caracterizó para la determinación de diferentes compuestos fenólicos en agua (alquifenoles, clorofenoles y nitrofenoles), compuestos especialmente apropiados para este formato ya que presentan grupos ionizables. Se llevó a cabo la optimización de todas las variables implicadas en el proceso (pH de la fase donadora y aceptora, fuerza iónica, velocidad y tiempo de agitación, y el volumen de muestra), obteniéndose bajos límites de detección (entre 82.1 ng/L para el fenol y 452 ng/L para el 2,4,5-triclorofenol) con una precisión mejor del 8 % (expresada como desviación estándar relativa).

Si comparamos los resultados obtenidos para los cinco clorofenoles comunes en función del formato de microextracción en fase líquida con membrana agitada empleado (**Tabla 1**), y teniendo en cuenta que se han usado sistemas instrumentales diferentes para cada caso (para el formato de dos fases GC/MS y para el de tres fases HPLC/UV), se puede afirmar que los límites de detección son mucho mejores cuando se emplea el formato de dos fases, pero esto se atribuye principalmente a la sensibilidad que proporciona el sistema instrumental. En cambio, si se comparan los factores de enriquecimiento, la preconcentración alcanzada por el formato de tres fases es mucho mayor a pesar de que la preconcentración teórica sea menor como demuestran las recuperaciones de extracción absolutas. La precisión de los resultados es comparable en ambos casos.

**Tabla 1**. Comparación de la microextracción líquida con membrana agitada en sus modalidades de 2 y 3 fases empleando varios clorofenoles como analitos modelo.

| Analito | 2 FASES | | | | 3 FASES | | | |
|---|---|---|---|---|---|---|---|---|
| | DER[a] | LDM[b] | FP[c] | FR[d] | DER[a] | LDM[b] | FP[c] | FR[d] |
| 3-clorofenol | 7.7 | 22.9 | 181 | 30.2 | 5.7 | 200.0 | 395 | 98.7 |
| 4-clorofenol | 8.6 | 20.0 | 166 | 27.7 | 8.0 | 284.2 | 317 | 79.2 |
| 2,6-diclorofenol | 8.7 | 15.8 | 296 | 49.3 | 7.8 | 381.8 | 363 | 90.8 |
| 3,4-diclorofenol | 5.0 | 15.0 | 316 | 52.7 | 5.4 | 363.6 | 359 | 89.6 |
| 2,4,5-triclorofenol | 7.2 | 14.8 | 246 | 41.0 | 6.7 | 452.6 | 168 | 42.0 |

[a] DER, desviación estándar relativa en porcentaje.
[b] LDM, límite de detección del método en ng/L.
[c] FP, factor de preconcentración.
[d] FR, factor de recuperación en porcentaje.
[e] LD, límite de detección en ng/L.

## 3. NUEVOS DESARROLLOS

La microextracción líquida con membrana agitada se emplea para mejorar la sensibilidad de las determinaciones y la selectividad de las mismas. Esto permite llevar a cabo determinaciones sensibles en muestras complejas, como es el caso de las muestras biológicas (orina, saliva), que se caracterizan por la presencia de un elevado número de interferentes y la baja concentración de los analitos a determinar. Esta selectividad viene proporcionada por las características intrínsecas de la membrana empleada, por el disolvente orgánico utilizado y por el gradiente de pH establecido a ambos lados de la membrana (tres fases). Así, por ejemplo, se ha llevado a cabo en el formato de tres fases y en combinación con el análisis con HPLC/UV, la determinación de seis antiinflamatorios no esteroideos (AINEs: indometacina, tolmetina, ketoprofeno, naproxeno, flurbiprofeno y fenbufeno) en orina humana [4]. Entre las ventajas de la metodología propuesta se pueden destacar la simplicidad, el bajo consumo de disolventes orgánicos, y los altos factores de enriquecimiento alcanzados. Los límites de detección obtenidos permiten la detección de AINEs en dosis terapéuticas, en muestras reales de orina. Aunque en su modo simple sólo permite la determinación de fármacos libres en las muestras, la hidrólisis previa de la muestra amplía la aplicabilidad de la metodología desarrollada a la determinación de los fármacos glucuronados.

Recientemente, se ha adaptado la microextracción líquida con membrana agitada al análisis de muestras biológicas de disponibilidad limitada, llevando a cabo la determinación de paracetamol en saliva humana [5]. Para esta aplicación, se modificó el diseño de la unidad de extracción con objeto de reducir el volumen de muestra a unos pocos mL (**Figura 3**). En la nueva unidad de extracción se incrementa la altura del elemento externo con respecto a la original, generándose una cámara superior donde se localiza la muestra. Por lo tanto, la muestra se encuentra integrada en la unidad de extracción, lo que permite además la agitación simultánea de varias unidades en el vórtex.

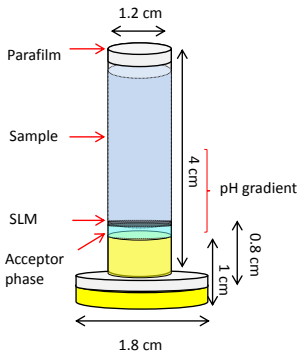


**Figura 3.** Elementos básicos de la unidad de microextracción con membrana agitada para el procesamiento de pequeños volúmenes de muestra.

## 4. CONCLUSIONES

La microextracción líquida con membrana agitada es una técnica sensible y selectiva, con un bajo consumo de disolventes orgánicos, que permite llevar a cabo el análisis de muestras complejas de forma sencilla y económica. Además, resulta ser una técnica muy versátil, puesto que puede seleccionarse en función del problema analítico, el formato (dos o tres fases), las fases implicadas en el proceso y la membrana. Finalmente puede acoplarse con diferentes técnicas instrumentales.

## 5. PERSPECTIVAS FUTURAS

Las técnicas que integran la extracción y la agitación han seguido una evolución continua en los últimos años [6]. Entre ellas, la extracción con membrana agitada ha sido especialmente empleada en el análisis medioambiental. Además, las características

inherentes de los nuevos desarrollos, hacen de esta técnica un instrumento prometedor para el aislamiento y la preconcentración de drogas en muestras biológicas, incluyendo aquellas de disponibilidad limitada. En este sentido, las futuras investigaciones deberían ir encaminadas a ampliar la aplicabilidad de este dispositivo de extracción a otras matrices biológicas como la sangre, el plasma, el semen o el sudor.

El material al partir del cual se han diseñado las unidades de extracción, PTFE, puede dar lugar a adsorciones indeseables. Por lo tanto, sería conveniente el estudio de nuevos dispositivos de extracción construidos a partir de materiales inertes.

La automatización es un punto débil de la técnica que limita su uso en el análisis rutinario. La potencial automatización de la técnica, incluso su integración con instrumentos analíticos, será una futura línea de investigación.

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**ANEXO C**

# Presentación de comunicaciones a congresos

**IX Jornadas Doctorales Andaluzas (JDA)** *(Menjibar, Jaén, 2011).*

- Comunicación oral y póster titulados "Innovaciones en técnicas de microextracción" M. Roldán-Pijuán, M. C. Alcudia-León, R. Lucena, S. Cárdenas, M. Valcárcel.

**II Congreso Científico de Investigadores en Formación. I Congreso Científico de Investigadores en Formación en Agroalimentación** *(Córdoba, 2012).*

- Comunicación oral titulada "Innovaciones en técnicas de tratamiento de muestra que integran la extracción y la agitación", M. Roldán-Pijuán, M. C. Alcudia-León, R. Lucena, S. Cárdenas, M. Valcárcel.

**Reunión del Grupo Regional Andaluz de la Sociedad Española de Química Analítica (GRASEQA)** *(Málaga, 2012).*

- Comunicación oral y póster titulados "Determinación de compuestos orgánicos volátiles en agua mediante microextracción con frita agitada acoplada a cromatografía de gases/espectrometría de masas", M. Roldán-Pijuán, M. C. Alcudia-León, R. Lucena, S. Cárdenas, M. Valcárcel.

**III Congreso Científico de Investigadores en Formación de la Universidad de Córdoba. II Congreso Científico de Investigadores en Formación en Agroalimentación. Universidad de Córdoba** *(Córdoba, 2013)*.

- Comunicación oral titulada "Microextracción líquida con membrana agitada para el análisis de muestras biológicas de disponibilidad limitada", M. Roldán-Pijuán, M. C. Alcudia-León, R. Lucena, S. Cárdenas, M. Valcárcel.

**XVII Euroanalysis** *(Varsovia, Polonia, 2013).*

- Póster titulado "Novel planar format extraction/stirring approaches" M. Roldán-Pijuán, M. C. Alcudia-León, R. Lucena, S. Cárdenas, M. Valcárcel.
- Poster titulado "Stir-membrane liquid microextraction for the determination of paracetamol in human saliva samples" M. Roldán-Pijuán, M. C. Alcudia-León, R. Lucena, S. Cárdenas, M. Valcárcel.

**XIII Reunión de la Sociedad Española de Cromatografía y Técnicas Afines (SECyTA)** *(Tenerife, 2013).*

- Stir-membrane solid-liquid-liquid microextraction for the determination of parabens in lyophilized human breast milk samples. R. Rodríguez-Gómez, M. Roldán-Pijuán, R. Lucena, S. Cárdenas, A. Zafra-Gómez, O. Ballesteros, A. Navalón, M. Valcárcel.

**Reunión del Grupo Regional Andaluz de la Sociedad Española de Química Analítica (GRASEQA)** *(Baeza, Jaén 2014).*

- Póster titulado "Microextracción en fase sólida basada en disco agitado modificado con nanocuernos de carbono oxidados de pared simple: aplicación para la determinación del disruptor endocrino benzofenona-3", M. Roldán-Pijuán, R. Lucena, S. Cárdenas, M. Valcárcel.
- Póster titulado "Disco agitado modificado con grupos octadecil para la microextracción en fase líquida de triazinas en agua", M. Roldán-Pijuán, R. Lucena, M. C. Alcudia-León, S. Cárdenas, M. Valcárcel.
- Comunicación oral titulada "Microextracción sólido-líquido-líquido con membrana para la determinación de parabenos en muestras de leche materna mediante cromatografía de líquidos acoplado a espectrometría de masas", R. Rodríguez-Gómez, M. Roldán-Pijuán, R. Lucena, S. Cárdenas, A. Zafra-Gómez, O. Ballesteros, A. Navalón, M. Valcárcel.

**V Encuentro sobre Nanociencia y Nanotecnología de Investigadores Andaluces (NANOUCO)** *(Córdoba, 2015).*

- Poster titulado: "Discos de borosilicato modificados con nanocuernos de carbono oxidados", M. Roldán-Pijuán, R. Lucena, M. C. Alcudia-León, S. Cárdenas, M. Valcárcel.